JOURNAL OF AGRICULTURAL RESEARCH

VOL. 45 WASHINGTON, D. C., SEPTEMBER 1, 1932 No. 5

RELATION OF COMMERCIAL HONEY TO THE SPREAD OF AMERICAN FOULBROOD[1]

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INTRODUCTION

The relation and importance of commercial honey to the spread of American foulbrood of bees has occupied the attention of the beekeeping industry more or less prominently for many years. The theory has been promulgated that honey which has not come from disease-free apiaries is dangerous because of the possibility of its disseminating American foulbrood. A few States and at least one foreign country require that honey intended for interstate shipment be accompanied by a certificate from the bee inspector of the State in which the honey originated to the effect that such honey was produced in apiaries free from American foulbrood.

It is a well-established fact that honey taken directly from the combs of the brood chambers of colonies affected by American foulbrood is capable of producing the disease if fed to healthy colonies. Since commercial beekeeping practice bans the extracting of honey from the brood nest, it is difficult to understand how heavily infected honey, in large quantities, could get on the market. Whether honey from supers that have been on colonies affected with American foulbrood is of serious importance in transmitting the disease is still open to question. White (*30, p. 35*)[3] says: "The likelihood that the disease will be transmitted by combs from diseased colonies, which contain honey but no brood, probably is frequently overestimated." On the other hand, Millen (*23*) found that combs built from foundation and completely filled above an excluder with honey from colonies that had been destroyed by American foulbrood produced disease in all of 10 colonies made from package bees to which one comb each of the honey had been given. Corkins (*8*) expressed the belief, as a result of preliminary studies, that "Extracted honey produced above an excluder in a colony in the early stages of American foulbrood is insignificant in the spread of this disease through commercial honey." The conflicting nature of these observations emphasizes the need for further research before the certification of honey is required as a means of alleviating the foulbrood situation.

In both animal and plant disease bacteriology it is known that pathogenic microorganisms may vary considerably, even within indi-

[1] Received for publication Feb. 1, 1932; issued September, 1932.

[2] For advice and assistance the writer is indebted to Profs. C. L. Corkins and C. H. Gilbert, of the University of Wyoming; Prof. R. G. Richmond, deputy State entomologist, apiary investigations, Colorado Agricultural College; H. Rauchfuss, of Englewood, Colo.; N. L. Henthorne, of Greeley, Colo.; and C. H. Ranney, of Lander, Wyo. Appreciation is also expressed for the many courtesies extended by H. C. Hilton, supervisor of the Medicine Bow National Forest.

[3] Reference is made by number (italics) to Literature Cited, p. 284.

vidual species, in virulence and in ability to produce disease. Furthermore, as stated by Zinsser (*31, p. 188–189*)—

> Whether or not infection occurs depends also upon the *number of bacteria* which gain entrance to the animal tissues. A small number of bacteria, even though of proper species and of sufficient virulence, may easily be overcome by the first onslaught of the defensive forces of the body. Bacteria, therefore, must be in sufficient number to overcome local defenses and to gain a definite foothold and carry on their life processes, before they can give rise to an infection. The more virulent the germ, other conditions being equal, the smaller the number necessary for the production of disease. The introduction of a single individual of the anthrax species, it is claimed, is often sufficient to cause fatal infection; while forms less well adapted to the parasitic mode of life will gain a foothold in the animal body only after the introduction of large numbers.

In the case of American foulbrood the quantity of infectious material that honey must carry in order to produce disease in a colony has never been determined. White (*30, p. 20, footnote 1*) states, in connection with inoculating healthy colonies experimentally with *Bacillus larvae:*

> It was found that less than one scale is sufficient disease material to produce a considerable amount of disease in the colony. In some experiments one scale, therefore, might supply all the spores needed although the use of a somewhat greater quantity of material is advisable in most instances.

While infected honey no doubt does become mixed with disease-free honey, it is probable in many cases that, because of the practice of using large settling and storage tanks, infected honey would be so diluted with spore-free honey as to make the spore content insufficient to produce infection even if fed to healthy bee larvae. Therefore, one object of these investigations was to determine the minimum number of spores of *Bacillus larvae* in honey necessary to produce American foulbrood in healthy colonies of bees as correlated with the infectivity or spore content of the average commercial honey.

In order to obtain information relative to this subject, experiments were conducted in the apiary over a period of five years. In these experiments honey or sugar sirup with a known content of spores of *Bacillus larvae* was fed to healthy colonies and the minimum number of spores that would produce infection was determined. At the same time laboratory studies were carried on with cultures of spores of *B. larvae*, concerning certain growth phases of the organism, particularly the minimum number of spores that would produce vegetative growth on artificial culture media. Methods for demonstrating the presence or absence of spores of *B. larvae* in samples of commercial honeys were also investigated, and these honeys were studied in relation to their infectiousness as correlated with the spore-feeding experiments. These three phases of the investigation will be discussed in the order mentioned.

MINIMUM NUMBER OF SPORES OF BACILLUS LARVAE NECESSARY TO PRODUCE DISEASE IN HEALTHY COLONIES OF BEES

METHODS OF PROCEDURE

Location of Experiments

These investigations were started during the summer of 1926 in a small experimental apiary located about half a mile from the bee culture laboratory of the Bureau of Entomology at Somerset, Md. The location at Somerset was undesirable, however, because of its

close proximity to the apiary connected with the laboratory and to other privately owned colonies of bees, necessitating extreme precautions to prevent spread of the disease. In 1927 the experimental work was transferred to the Intermountain States bee culture field laboratory at Laramie, Wyo.[4] In Wyoming an ideal isolated location was found about 14 miles east of Laramie in the Medicine Bow National Forest, the nearest colonies of bees being at least 14 miles away and probably farther. Since this location is more than 8,000 feet above sea level, there is only a slight nectar flow from wild flowers, which assures the immediate use of any inoculated sirup fed to colonies of bees. In fact, after the middle of the summer it was found necessary in most cases to feed the experimental colonies with uninoculated sugar sirup in order to prevent starvation.

In 1927 and 1928 the colonies used for experimentation were located in two yards between a quarter and a half mile apart. The arrangement of the colonies in the two yards was such as to prevent drifting as much as possible. In 1929 and 1930, in order to limit still further the danger of transmission of disease because of drifting or robbing, 20 colonies were stationed in pairs, so arranged as to minimize the danger from drifting, in 10 isolated locations at least a quarter of a mile apart.

Make-up of Colonies

Five-frame nucleus hives were used for the spore-feeding experiments. The colonies were prepared either with two or three frames of brood, honey, and adhering bees taken from healthy colonies, together with a young laying queen, or, as in 1927, 1928, and 1929, by placing a 2-pound package of bees containing a laying queen on foundation or on combs containing honey from healthy colonies and feeding them sugar sirup. During a good honey flow these small colonies were allowed to build up in the apiary connected with the laboratory until they consisted of three or four frames of brood before they were moved to the isolated locations. The bees making up the colonies used for the feeding experiments from 1927 to 1930 at Laramie, Wyo., were all from the same general strain.

Material Used for Inoculation

Spores of *Bacillus larvae* were obtained from American foulbrood scales in combs taken from diseased colonies located in the States of Maryland, Iowa, and Wyoming. The strain used at Somerset, Md., was obtained from a sample sent to that laboratory for diagnosis. Two different strains were used at Laramie during 1927, 1928, and 1929, one obtained from a diseased colony in the experimental apiary belonging to the University of Wyoming and one obtained from a beekeeper at Lander, Wyo. In 1930 three other strains were used in the feeding experiments, one from Iowa and two from apiaries in Wyoming.

Preparation of Spore Suspensions

In preparing the spores for feeding to the healthy colonies, scales were removed from the combs by means of sterile forceps (the necessary precautions being taken against contamination) and placed in

[4] This laboratory is maintained cooperatively by the University of Wyoming and the U. S. Department of Agriculture.

a flask containing 50 c c of sterile water and glass beads. After the scales had softened in the water, the flask was shaken for one-half hour to insure complete maceration of the scales. The suspension was then filtered through two thin layers of sterile absorbent cotton into another sterile flask in order to remove any lumps or débris.

In preparing the stock suspensions of spores, at first 75 to 100 scales were taken by counting. Later it was found that the average American foulbrood scale weighs 0.0223 g. Therefore, the 100 scales for the stock suspensions were obtained by weight, the scales being weighed in a sterile covered glass dish before they were deposited in the flask of sterile water.

After the suspension had been filtered and tested for contamination and was ready for use, the number of spores per cubic centimeter was determined by the following method: By means of a blood-diluting pipette giving a dilution of 1 to 20, the spore suspension was diluted with a weak solution of carbol fuchsin and a drop placed in the counting chamber of a Helber bacteria-counting cell 0.02 mm deep and ruled in squares of 0.0025 mm^2 each.[5] With the use of two 15× eyepieces in a binocular microscope and a 1.8-mm oil-immersion objective, the spores in 25 squares of the Helber chamber were counted. Then by means of the formula

$$\frac{\text{Total spores counted} \times \text{dilution} \times 20{,}000 \times 1{,}000}{\text{Number of squares counted}}$$

the approximate number of spores per cubic centimeter in the suspension was determined.

Later this method was checked by the method of Breed and Brew (*2*) for counting bacteria in milk. With the aid of a binocular microscope having two 15× eyepieces and a 1.8 mm oil-immersion objective, the area of a circle etched on an ocular micrometer disk was determined by means of a stage micrometer. One one-hundredth cubic centimeter of a 1 to 100 dilution of the stock suspension of spores was placed on a glass slide on which 1 cm^2 had been ruled with a diamond pencil. This was mixed with a small loopful of carbol fuchsin stain and the whole spread over the 1 cm^2 of surface[5] and allowed to dry uniformly. The number of spores per cubic centimeter of the stock suspension was determined according to the formula

$$\frac{\text{Area 1 cm}^2}{\text{Area of circular field}} \times \frac{\text{total number of spores counted} \times \text{dilution} \times 100.}{\text{number of circular fields counted}}$$

These two methods were found to check fairly closely within the limits of the precision of the methods used in counting. Furthermore, by both methods it was found that in the majority of cases 100 scales in 50 c c of water give approximately 5,000,000,000 spores per cubic centimeter for each suspension made up in this way. Therefore, this number was used as a standard for making all dilutions.

[5] Mm^2 and cm^2 are the abbreviations for square millimeter and square centimeter, respectively, recently adopted by the Style Manual for United States Government printing.

After a considerable number of counts had been taken in making up several stock suspensions of spores, counting was eliminated and the spore content of the stock suspensions was standardized according to the method described by Gates (*11*, p. 114), as follows: "The opacity of a bacterial suspension is measured by the length of a column of the suspension required to cause the disappearance of a wire loop." An instrument known as a suspensiometer was used for this purpose. The use of this method saved considerable time and labor without appreciably affecting the precision of the counts. One liter of a 50 per cent solution of sugar in water was used as the standard quantity of inoculated sirup fed to each experimental colony. A series of dilutions of the original stock suspension containing 5,000,000,000 spores was made by adding different quantities of the spore suspension to 1 liter of sugar sirup. In this way the approximate total number of spores in each liter of sugar sirup to be fed to colonies of bees was known.

Method of Inoculating Colonies

In 1926 at Somerset, Md., the sugar sirup containing the various dilutions of spores was fed to the colonies by means of galvanized-iron troughs that were hung inside the hives after two combs had been removed. In these troughs sterile excelsior was placed for the bees to walk on in order to prevent them from drowning. This method was found unsatisfactory, however. At Laramie, Wyo., the sugar sirup containing the spores was first placed in Boardman feeders, but owing to the danger of robbing at the entrance of the hives, the method finally used was to invert the jars in holes bored in the hive covers. In this way any leakage into the hives was cleaned up by the bees without danger of causing robbing. To prevent the jars from being broken or knocked over, box covers were placed over them and fastened to the hive covers. Each colony was usually inoculated only once with an individual dilution of spores. Duplicate colonies were inoculated with each dilution of spores. Uninoculated check colonies were placed among those that were inoculated.

Primary Observations

Observations of the condition of the brood were made at least once a week, and sometimes oftener, after the colony was given the liter of inoculated sirup. In 1926 at Somerset, Md., as soon as diseased larvae appeared in a colony, the colony was killed and at once removed from the apiary. Because of the isolated location near Laramie, Wyo., the colonies were left until the end of the brood-rearing season, when final observations were made.

The results of the spore-feeding experiments are shown in Table 1.

TABLE 1.—*Results of spore-feeding experiments* [a]

[Duplicate colonies of bees (A and B) were used in the first 4 years, and triplicate colonies (A, B, and C) in 1930]

Total number of spores fed	Extent of foulbrood in—																			
	1926		1927				1928, repeat		1928				1929				1930, final			
			During season		Final				During season		Final		During season		Final					
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	C	
5,000,000,000	+	?+																		
2,500,000,000	+	+							+	+	+	+								
1,000,000,000	+	+							+	+	−*	+								
750,000,000	+	+																		
500,000,000	+	+	+		−*				+	+	−*	+	+	+						
350,000,000	+	0	+		+				+		+									
200,000,000	+	?+	+	+	−*	−*	+													
175,000,000			+	?+	+	−*		+												
150,000,000			?+	+	−*	−*	−	−												
125,000,000			0	?+	0	−*	0	−												
100,000,000	0	0	0	?+	0	−*	0	−	+	+	−*	+								
75,000,000			+	?+	−*	−*		+	0	+	0	+	+	0	+	0					
50,000,000	0	0	0	0	0	0			0	+	0	−*	0	+	0	−*	0	0	0	
25,000,000													0	0	0	0	0	0	0	
10,000,000	0	0											0	0	0	0				
5,000,000													0	0	0	0				
2,500,000													0	0	0	0				
1,500,000													0	0	0	0				
500,000													0	0	0	0				
100,000													0	0	0	0				
Controls	1+,12−0		1+,2−0		1+,2−0		1−0		1+,3−0		1+,3−0		8−0		8−0		2−0			

[a] +, Positive American foulbrood; ?+, probable American foulbrood, very slight and unconfirmed and disappearing by end of brood-rearing season; 0, no disease found during season; −*, disease cleaned out by end of brood rearing; −, no recurrence in second season.

In 1926 a total of 200,000,000 spores fed to a colony was the smallest number that produced disease; in 1927, on the other hand, 75,000,000 was the smallest number. However, in the latter year the spores were obtained from another locality in which environmental conditions were quite different. In an effort to obtain check results, the feeding experiments were repeated in 1928. Through an error in making up the spore dilutions, which was not discovered until too late for rectification, no colony received less than 50,000,000 spores. This season one colony of the pair receiving an inoculation of 50,000,000 spores became infected. The feeding experiments were repeated again in 1929, with dilutions of spores from 75,000,000 down to 100,000—considerably less than the minimum number in 1928. Again only one colony of the pair receiving a total of 50,000,000 became infected. As a result of two years' experiments this was found to be the apparent minimum number of spores of *Bacillus larvae* capable of producing infection when fed in 1 liter of sugar sirup. In 1930 spores from three different localities were fed in duplicate to six healthy colonies in dilutions of 50,000,000 and 25,000,000 without producing disease.

It is therefore apparent that a certain minimum number or mass of spores is required to start the initial action capable of producing American foulbrood in healthy larvae. Under the conditions of these experiments this minimum number was approximately 50,000,000 spores of inoculum per liter of sirup.

SECONDARY OBSERVATIONS

During the first three years of the experiments, or previous to 1929, at which time the experimental colonies were isolated in pairs, certain of the uninoculated control colonies developed disease, 1 out of 13 in

1926, 1 out of 3 in 1927, and 1 out of 4 in 1928. It was assumed that the disease was probably not spread by robbing, since no active robbing was observed at any time. In practically every case where a control colony became infected, it was so located in relation to the inoculated colonies that drifting of young nurse bees during play flights could account for the spread of the disease, in one or two cases quite definitely so. In 1929 all eight uninoculated colonies, although they were not located with the inoculated colonies but were within robbing range of all, remained free from disease. The prevention of drifting apparently eliminated the casual spread of disease.

Occasionally a colony of bees affected with American foulbrood will try to clean out the diseased remains, often removing parts of the scales and sometimes actually tearing a comb down to the midrib in order to do this. White (*30, p. 34–35*) states:

> There is considerable evidence to support the belief that occasionally in cases of light infection the disease may disappear unaided by treatment. * * * It should be emphasized that such a course for the disease, if it occurs at all, is unusual. Although American foulbrood spreads more or less rapidly within an infected colony, the fact remains that it frequently does not.

Lineburg (*16*) in 1925 reported that in two colonies which were diseased in the spring the disease apparently disappeared later in the season. Three colonies were divided and used for making increase in June and July, but all remained free from disease, at least until the end of that season. Further observations were not reported. Corkins (*8*) in 1928 reported five colonies which were given combs containing scales of American foulbrood at the beginning of the honey flow of 1927 and developed no disease up to July 10, 1928. Two other colonies were observed to have cleaned out the disease and remained healthy for an entire season. However, during the several years of his experimental work on American foulbrood, the writer never observed a colony in which the disease was permanently cleaned out until 1927. In that year, of 16 colonies inoculated with various dilutions of spores, 4 colonies, 2 of which received more than the probable minimum dose causing infection, showed no disease during the season. The disease completely disappeared by the end of brood rearing in 10 of the 12 other colonies that had showed either positive or probable disease some time during the summer. In 1928 package bees were placed on the combs of seven of these colonies that had apparently cleaned out the disease during the previous summer and on two that had been inoculated with presumably a sufficient number of spores but which had remained healthy. Three of the seven developed disease again the second season, while four remained healthy during the entire season. Neither of the two inoculated colonies that had remained free from disease in 1927 developed it in 1928. Of the 11 colonies inoculated in 1928 that developed disease, 4 cleaned up the disease by the end of the brood-rearing season and 2 inoculated colonies showed no disease. In 1929, 1 of the 2 colonies developing disease cleaned up by the end of the brood-rearing season, making a total of 15 cases in which the disease was cleaned up by the end of brood rearing. Two of the colonies inoculated with the minimum infectious dose or more showed no disease during that summer.

It is possible that, in the high altitude of Laramie, and in similar places where the air is very dry, the scales of American foulbrood

become dried without adhering so tenaciously to the cell walls as they do in more humid climates at lower altitudes. These observations indicate the necessity of further work on the resistance of bees to the disease and variation in virulence of different strains of the organism.

INOCULATION OF INDIVIDUAL BEE LARVAE WITH DEFINITE NUMBERS OF SPORES OF BACILLUS LARVAE

In the light of the results of the foregoing experiments, in which colonies were inoculated with presumably a quantity of spores sufficient to produce infection but in which no disease developed, the question arises as to what became of the spores in the sugar sirup, some of which presumably were fed to healthy larvae. In those colonies developing disease that received a minimum number of spores, how many spores did each larva developing the disease receive? In order to obtain information on these points, a preliminary series of experiments was planned in which individual larvae were inoculated with known numbers of spores.

Toumanoff (*29*) reports that he was unable to cause infection by giving individual larvae a drop of a rich emulsion of a culture of *Bacillus larvae* in salt solution. He found that many of the larvae so treated were removed from the cells by the bees, and those remaining failed to develop disease. He further found that larvae given only uninoculated salt solution were also removed in the same way. Therefore, in the present experiments sugar sirup was used instead of salt solution. In a comb from a healthy colony containing numerous coiled larvae, a drop of an uninoculated 50 per cent solution of sugar in water was placed in each cell containing a larva, as near the mouth parts of the larva as possible. The rim of each cell so treated was marked with a paint consisting of 1 part of liquid white shellac, 1 part of a paint pigment, and 4 parts of ethyl alcohol. The sugar sirup was slightly colored with water-soluble eosin in order to aid in determining the effect. Frequent observations showed that practically all larvae that were fed this colored sugar sirup developed normally and were sealed over, the pigment markings still being present on the edges of the cappings. In most of the cells a residue of colored sirup could be observed for several hours after the larvae had fed.

A series of 5-frame nuclei was prepared, each containing one or two combs having a large number of unsealed larvae. A set of dilutions of spores was made from a stock suspension with a sterilized 50 per cent sugar sirup in such a way that each 0.01 c c of the dilution would contain an approximate known number of spores, as indicated in Table 2. Sterilized 2 c c Luer tuberculin hypodermic syringes graduated in 0.01 c c, the needles of which had been blunted, were used in inoculating the cells containing coiled larvae. Fifty or more coiled larvae at least 4 days old were each given 0.01 c c of a dilution of spores, each dilution being given to larvae in one comb in a separate colony, and the cells so inoculated were distinctively marked. A few larvae that had just been sealed also were inoculated by puncturing the capping with the inoculating needle and depositing the 0.01 c c in the cell. Observations were taken at the end of 24 hours and at frequent intervals thereafter until the end of the brood-rearing season.

TABLE 2.—*Results of inoculation of individual healthy bee larvae with known numbers of spores of bacillus larvae, 1930*

Spores fed each larva in 0.01 cubic centimeter of dilution	First inoculation, July 14		Second inoculation, Aug. 1			Third inoculation, Aug. 19							Fourth inoculation, Aug. 21							
	Colony No.	Larvae developing disease Aug. 1	Colony No.	Larvae developing disease		Colony No.	Length of time isolated from nurse bees	Effect on larvae of being isolated from nurse bees	Larvae developing disease				Colony No.	Length of time isolated from nurse bees	Effect on larvae of being isolated from nurse bees	Larvae developing disease			
				Aug. 19	Oct. 7				Aug. 26	Sept. 9	Sept. 19	Oct. 7				Aug. 26	Sept. 9	Sept. 19	Oct. 7
Number		*Number*		*Number*	*Number*		*Hours*		*Number*	*Number*	*Number*	*Number*		*Minutes*		*Number*	*Number*	*Number*	*Number*
50,000,000	.....	.....	.....	.....	.....	12	1	Larvae removed	0	0	0	0	12	5	Larvae removed	0	0	0	0
25,000,000	.....	.....	.....	.....	.....	13	1	....do	0	0	0	0	13	10	....do	0	0	0	0
10,000,000	.....	.....	.....	.....	.....	20	¾	Larvae not removed	0	[1] 13	.....	.....	20	15	Larvae not removed	0	[*] 9	.....	.....
7,500,000	.....	.....	.....	.....	.....	15	1	Three-fourths of larvae removed.	0	0	0	0	15	20	....do	0	0	0	0
5,000,000	.....	.....	14	0	0	4	¾	One-half of larvae removed.	0	0	0	0	4	30	One-half of larvae removed.	0	0	0	0
1,000,000	.....	.....	34	0	0	16	1	Larvae not removed	0	0	0	0	.....	.....	.....	.....	.....	.....	.....
500,000	.....	.....	16	0	0	22	1	....do	0	0	0	0	.....	.....	.....	.....	.....	.....	.....
250,000	.....	.....	18	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
100,000	.....	.....	22	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
75,000	.....	.....	26	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
50,000	.....	.....	28	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
25,000	.....	.....	4	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10,000	.....	.....	15	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
7,500	.....	.....	20	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5,000	12	0	12	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1,000	13	0	13	0	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
750	14	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
500	15	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
250	18	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
100	22	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
75	26	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
50	28	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
25	4	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
10	15	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
5	20	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
1	34	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
0	32	0	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

[*] Number of cells showing American foul-brood remains.

In the first series of inoculations the number of spores fed each larva ranged from approximately 5,000 down to 1. None of the larvae inoculated developed disease. (Table 2.) Later a second series of inoculations was made. The same colonies were used because of the limited number available, but the larvae inoculated were in a different comb in each colony and a different color was used to mark the cells. In these inoculations the number of spores fed ranged from 5,000,000 down to 1,000 per larva. No disease developed from this set of inoculations.

It was thought possible that the nurse bees might be removing most, if not all, of the inoculated sugar sirup before the larvae had had time to ingest a sufficient number of spores to bring about infection. Therefore, in a third series of experiments each inoculated comb was placed in a screen-wire queen-nucleus introducing cage, and this cage was put back in the colony for periods ranging from one-half to one hour before the unprotected comb was replaced in the colony, thus theoretically giving the larvae time to ingest some of the sugar sirup before the nurse bees had access to the inoculated cells. In these tests the larvae were kept from the bees so long that many of them, becoming hungry, were starting to crawl from the cells. The number of spores fed ranged from 50,000,000 down to 500,000 per larva. Twenty-four hours after the larvae were fed it was found that all receiving 50,000,000 and 25,000,000 spores had been removed from the cells, while those receiving a smaller number of spores were either partly removed or remained in the cells, according to the strength of the dilution and the length of time that the larvae were kept away from the nurse bees. (Table 2.)

Two days later another set of larvae was inoculated with the same dilutions as were previously used for these colonies but on the other side of the same combs. In this series the combs were kept away from the bees for periods ranging from 5 minutes for the heaviest dilution to 30 minutes for the weakest. Again all the larvae receiving the 50,000,000 and 25,000,000 spores were removed, while those receiving the 5,000,000, which were kept from the bees for half an hour, were partly removed, and those receiving 7,500,000 or 10,000,000 were not removed. Apparently there are two factors concerned in the removal of the larvae—the length of time they are kept away from the bees and the amount of foreign matter in the sirup, as indicated by the spore content, that is given to the larvae.

The results of the last two series of inoculations showed that in the colonies in which the larvae were not removed, or were not entirely removed, several larvae in the colony receiving 10,000,000 spores per larva developed disease, while those in the colonies receiving a smaller number remained healthy. (Table 2.) This work should be repeated with a different colony for each set of inoculations, although apparently the disease did not spread in the colonies used. Only one colony of the entire number developed disease. Although a certain degree of success was obtained, these results seem to bear out Toumanoff's (*29*) conclusion that the artificial infection of individual larvae is not brought about so easily as one had been in the habit of believing. Apparently, also, a considerable number of spores are necessary to establish an infection under these conditions.

MINIMUM NUMBER OF SPORES OF BACILLUS LARVAE PRODUCING VEGETATIVE GROWTH ON ARTIFICIAL CULTURE MEDIA

Bacteria are known to pass through a definite cycle of growth, particularly when cells from an old culture are transferred to fresh culture media. The growth stages have been described by Buchanan (*3; 13, Ch. V*), Henrici (*12*), and Winslow (*13, Ch. VI*) somewhat as follows: The initial stationary phase during which no growth takes place; the logarithmic phase when the organisms begin to divide, slowly at first but gradually accelerating; and so on through the complete cycle of growth. Henrici (*12, p. 21, 24*) has observed that—

> Various factors, as temperature; the size, the age, and previous history of the inoculum; and the composition and nutrient value of the medium, influence the growth curves of bacteria. * * * Of the various factors which influence the rate of growth and form of the growth curve, the initial number of cells introduced into a unit volume of medium seems to be one of the most important.

Robertson (*25*), in studies of cultures of certain protozoa, has shown that growth seems to be stimulated by the presence of other cells of the same type. This characteristic has been described at various times as mass action or communal activity.

Early in 1929, in conjunction with the spore-feeding experiments in the apiary, an investigation was started to determine whether there is a similar manifestation of mass action in the vegetative growth of spores of *Bacillus larvae* on artificial culture media. In a preliminary paper on this subject the writer (*27, p. 456*) made the following observations: Starting with a seeding of 5,000,000,000 spores of *B. larvae* on a suitable slanted solid culture medium, it was found at the end of 48 hours' incubation at 37° C. that growth had occurred in the original and in a diluted seeding containing 60,000,000 spores, but not in one containing 50,000,000 spores. Growth occurred in a diluted seeding containing only 5,000,000 spores after six days' incubation, and in one containing 700,000 spores after 10 days' incubation. (Table 4, Group 1.) These observations indicated that a certain initial mass of spores is necessary to start vegetative growth. Furthermore, although the growth results were rather irregular owing to the comparatively small number of cultures made, they seemed to show that, within certain limits, the smaller the seeding the longer the incubation period necessary to obtain germination of the spores and vegetative growth. From this preliminary work it was assumed that the lower limits of dilution of the stock suspension that would give growth on longer incubation had not been reached.

Ahrens (*1*) has observed, in cultural studies of scales treated with formalin solution for different lengths of time, that growth may occur in cultures from such scales after varying periods of incubation up to 30 days, depending on the length of treatment and the percentage of formalin in the solution. Burnside (*7*) states, in connection with studies of disinfection of American foulbrood combs by fumigation with formaldehyde gas, that "it is probable that if scales had been washed and the incubation period increased, growth of *Bacillus larvae* would have been obtained in some instances when negative results were recorded."

Therefore, a single trial series of cultures was run (No. 7, Table 4), the total incubation period being 30 days. Results from this set of cultures showed that in some cases growth was obtained after 30

days' incubation where no growth was observed after 10 days' incubation. Work on this phase of the problem was continued during the summer and fall of 1930. Several sets of cultures were made in which *Bacillus larvae* from eight different localities were used in a series of seedings with a decreasing number of spores for each lot of the organism and all incubated for 30 days. (Table 4, Group 2.)

METHODS OF PROCEDURE

Culture Media

A culture medium was used similar to that employed by the writer in the preliminary experiments (*27*) and also in earlier cultural work with *Bacillus larvae* (*26*)—that is, a combination of the medium made of yeast-extract and egg-yolk suspension and the carrot-extract medium of Lochhead (*18*). The yeast-carrot extract medium was prepared as follows:

(A)	Dried yeast	grams	10
	Peptone	do	10
	Buffer (sodium glycerophosphate)	do	2.5
	Water (distilled)	cubic centimeters	500

This solution was heated in flowing steam for one-half hour and, after a tablespoonful of siliceous earth had been added to assist in the filtration and clarification, it was filtered through filter paper on a perforated porcelain funnel with suction.

(B) Two hundred grams of cleaned carrots was macerated in a meat grinder, added to 500 c c of distilled water, and allowed to stand for at least 30 minutes, preferably longer. The macerated carrot was removed by filtration through fine muslin, as much liquid as possible being squeezed from the mass. The filtrate was then clarified by the addition of siliceous earth and filtration in the same manner as the yeast-extract medium.

(C) The final base medium was prepared by mixing 500 c c of A with 200 c c of B and adding 700 c c of a 3 per cent solution of washed agar.

The reaction of the medium was so adjusted that when 2 c c of sterile egg-yolk suspension, prepared as described in a previous paper (*26*), was added to 10 c c of the yeast-carrot extract base medium by means of the apparatus shown in Figure 1, and described previously (*26*), the pH value was 6.8. The medium was then sterilized in the autoclave at 15 pounds' pressure (sea level) for 15 minutes. After it had cooled to 45° C., 20 drops, or about 2 c c, of the sterile egg-yolk suspension was added to each tube of medium, mixed by shaking, and the medium was then allowed to solidify in a slanting position.

The Lochhead yeast-extract medium was tried without the addition of egg-yolk suspension, but although it gave good growth with the heavier seedings of spores, the combination medium was found to give more uniform germination and heavier vegetative growth with the more dilute seedings. The addition of the carrot extract, while possibly adding somewhat to the growth-producing qualities of the medium, served in these experiments as an indicator for vegetative growth because of the ability of *Bacillus larvae* to produce nitrite in the carrot-extract medium without the addition of potassium nitrate (*18*).

Preparation of Dilutions of Spores

The stock suspensions of spores of *Bacillus larvae* were made up as described earlier in this paper. A series of primary dilutions, each one-tenth of the preceding dilution, was then made up in sterile 125

c c flasks by adding 4 c c of a dilution to 36 c c of sterile water. The series of dilutions containing gradually decreasing numbers of spores per cubic centimeter to be used in inoculating the culture medium were then prepared as indicated in Table 4. Sterile burettes were used in adding the proper proportions of spore suspension or spore-suspension dilutions to the proper quantities of sterile water in sterile test tubes, in order to make up the desired series of dilutions containing approximately known numbers of spores.

Inoculation of Culture Medium

Swann has observed that in old cultures of anthrax a considerable percentage of spores are dead and therefore never germinate. Because of the possibility that some of the spores in the stock suspensions of *Bacillus larvae* might not be viable, an effort was made to determine the approximate proportions of viable and dead spores in the stock suspensions. Since the determination of viable spores of *B. larvae* by means of plate cultures is difficult because of the opaqueness of the special culture medium that is required, an attempt was made to determine the percentage of viable spores by the differential staining method of Burke (*4*) as modified by Koser and Mills (*15*). The procedure is as follows: A small quantity of the spore suspension is spread in a thin film on a slide and allowed to dry without heating. The slide, after immersion in a solution of carbol fuchsin at room temperature for two minutes, is washed in water and decolorized with absolute acetone for a few seconds, washed again, and immersed in Loeffler's alkaline methylene blue for two minutes, washed, dried, and examined. Very few solid-staining forms were observed in any of the suspensions examined, possibly one or two spores in several fields. It was therefore assumed that the number of nonviable spores could be considered as negligible and probably within the limits of the precision of the measurements as indicated by this procedure.

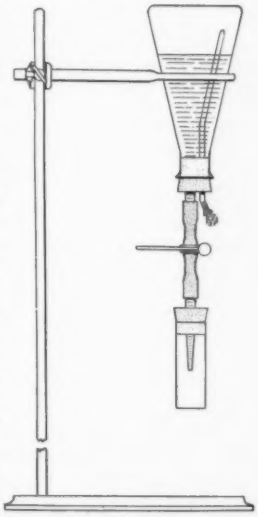

Figure 1.—Apparatus to replace pipetting of egg-yolk suspension

One cubic centimeter of each dilution was added to duplicate tubes of the slanted solid medium by means of sterile 1 c c pipettes, each cubic centimeter of inoculum containing an approximately known number of spores of *Bacillus larvae*. After inoculation the cultures were incubated at 37° C. In order to prevent the liquid in the tubes from drying out on long incubation, from time to time, as the water of condensation evaporated, 2 or 3 c c of sterile broth similar in composition to that of the base medium, without the egg, was added to each tube by means of the apparatus shown in Figure 1. A total of 556 cultures was made during this series of experiments.

Method of Making Observations

The culture tubes were incubated for 30 days at 37° C. Each tube was examined usually every 24 hours during this period. The presence or absence of vegetative growth was noted at each observation, and in cases of slight or doubtful growth the vegetative growth was checked both by microscopic examination of a stained smear and by testing for nitrite production in the culture medium by the sulphanilic acid and alpha-naphthylamine acetate test. After a large number of such observations had been made, it was found that vegetative germination of spores of *Bacillus larvae*, almost too slight to be seen, would give a definite pink color on the addition of the reagents.

Lochhead (*17, p. 14*) states:

> It was found, however, that ordinary nitrate-reducing species, such as *B. cereus* or *Es. coli*, which are able to form nitrites readily in nitrate media, were unable to produce nitrites in recognizable amount in the peptone-carrot media, though capable of doing so upon the addition of nitrates. *Bacillus larvæ* under the same condition readily forms nitrites without the addition of nitrate to the medium.

Despite this statement, a series of miscellaneous organisms was tested in standard nitrate broth, in carrot-extract broth, and on carrot-extract agar. Several organisms that commonly reduce nitrates and a few that do not were used. (Table 3.) Observations were made at short intervals during the first 24 hours. Most of these organisms gave positive nitrite tests within a few hours after inoculation in all the media used, but in the carrot-extract medium the nitrate had apparently disappeared in most cases after 24 hours' incubation, and in all cases after 48 hours. The same organisms on standard nitrate medium still gave positive tests after 48 hours' incubation. A positive nitrite test was obtained in cultures of *Bacillus larvae* that were incubated for 5 days and in one culture that was incubated for 4 days and then allowed to stand at room temperature for 16 days more before testing. Therefore, it appears probable—at least the results in Table 3 indicate—that in the case of many contaminating organisms having the power to reduce nitrite that might get into the culture tubes inoculated with spores of *B. larvae* the nitrite, if produced by the contaminating organism, would have disappeared after 48 hours' incubation, leaving contamination to be determined by gross appearence of the culture and microscopic examination. Nevertheless, in order to be sure that contaminating growth of any kind was not giving erroneous results with the nitrite test when this was used alone, any suspicious-looking growth in the culture tubes was examined under the microscope before it was tested with the reagents for nitrite production. Even though a positive nitrite test might be observed in some cases, the contaminations were recorded only as such.

Observations and Results

In no instance was positive growth obtained in cultures inoculated with less than 50,000 spores, even after 30 days' incubation, and growth with 50,000 spores was obtained from only two of the eight lots of spores used, namely, Nos. 19 and 23. (Table 4.) In the other six strains the minimum number of spores that produced positive growth ranged from 5,000,000 to 70,000.

TABLE 3.—*Nitrate reduction by various miscellaneous organisms in standard nitrate broth and carrot-extract media during different periods of time*[a]

Organism	Standard nitrate broth					Carrot-extract broth							Carrot-extract agar					
	3 hours	6 hours	8 hours	24 hours	48 hours	3 hours	6 hours	8 hours	10 hours	14 hours	16 hours	24 hours	3 hours	6 hours	24 hours	48 hours	5 days	20 days
Escherichia communior	3+	4+	4+	4+	4+	2+	−	−	−	−	−	−	+	+	−	−	.....	.....
Escherichia coli	−	3+	4+	4+	4+	−	+	−	−	−	−	−	+	+	−	−	.....	.....
Eberthella typhi	−	3+	4+	4+	4+	−	−	−	−	−	−	−	+	±	−	−	.....	.....
Alcaligines fecalis	+	3+	4+	±	−	Tr.	2+	2+	+	+	−	−	2+	+	+	−	.....	.....
Aerobacter aerogenes	3+	4+	4+	4+	4+	+	−	−	−	−	−	−	Tr.	Tr.	±	−	.....	.....
Pseudomonas aeruginosa	−	3+	4+	−	−	+	2+	2+	+	±	−	−	+	+	+	−	.....	.....
Serratia marcescens	−	3+	4+	4+	4+	−	+	−	−	−	−	−	Tr.	Tr.	+	−	.....	.....
Staphylococcus albus	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	.....	.....
Bacillus subtilis	.....	.....	.....	−	−	.....	.....	.....	.....	.....	.....	−	.....	.....	−	−	.....	.....
Bacillus mesentericus	.....	.....	.....	−	−	.....	.....	.....	.....	.....	.....	−	.....	.....	−	−	.....	.....
Bacillus larvae	.....	.....	.....	2+	4+	.....	.....	.....	.....	.....	.....	2+	.....	.....	2+	4+	4+	4+

[a] +, 2+, 3+, and 4+ indicate relative degrees of reaction; Tr. indicates trace; − indicates no reaction; ± indicates that the reaction was doubtful.

TABLE 4.—*Vegetative growth in cultures of Bacillus larvae with varying dilutions of a stock suspension*

Method of making dilutions			Spores per cubic centimeter inoculated ($\times 10^3$)	Strains of bacillus larvae *													
				Group 1 (preliminary experiments)						Trial series	Group 2						
Primary dilution taken	Quantity of primary dilution taken	Water added		No. 1	No. 4	No. 5	No. 6A	No. 6A2	No. 6D	No. 7	No. 12	No. 13	No. 14	No. 15	No. 19	No. 20	No. 21	No. 23
	Cubic centimeters	*Cubic centimeters*																
	Original (A)	0	*Number* 5,000,000	+2 +2	+2 +2	+2	+2	+6	+10	+5	+2 +2	+2 =2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +1	+2 +2
	8	2	4,000,000			+2												
A	6	4	3,000,000			+2												
	4	6	2,000,000			+2												
	2	8	1,000,000			+2												
	1 (B)	9	500,000	+2 +2	+2 +2	+2	+2	+6	+10	+5	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2	+2 +2
	8	2	400,000		+2	+2	+2											
	6	4	300,000		+2	+2	+2											
	4	6	200,000		+2	+2	+2											
B	2	8	100,000		±2?	+2	+2											
(1A+9H_2O)	1.8	8.2	90,000			+2	+2											
	1.6	8.4	80,000			+2	+6											
	1.4	8.6	70,000			−2	+6											
	1.2	8.8	60,000			−2	+2											
	1.0 (C)	9.0	50,000	±2±2	±2?−2	−2	−6	+6	+10	+5+30	+3 +3	+3 +3	+3 +3	+2 +2	+2 +2	+4 +4	+5 +5	+4 +4
	8	2	40,000			−2	−6	−6	+10	+10+10	+3 +3	+3 +5	+4 +4	+2 +2	+2 +2	+4 +4	+5 +5	+4 +4
	6	4	30,000			−2	+6	−6	+10	+10+15	+3 +3	+5 +5	+4 +4	+2 +2	+3 +3	+4 +6	+5 +5	+4 +4
	4	6	20,000			−2	−6	+6	+10	+10+30	+3 +3	+5 +5	+4 +4	+3 +3	+3 +3	+4 +6	+7 +7	+4 +4
C	2	8	10,000			−2	−6	+6	+10	−30−30	+7 +8	+5 +7	+6 ×	+3 +3	+6 ×	+6 +6	+7+14	+4 +4
(1B+9H_2O)	1.8	8.2	9,000			−2	+6	+6	−10	−10+30				+4 +4	+6 +6	+6 +6	+9+27	+4 +4
	1.6	8.4	8,000			−2	−6	+6	+10	−30−30				+4 +4	+6+16	+6+11	−27−30	+4 +4
	1.4	8.6	7,000			−2	−6	+6	−10	+10−10				+4 +4	−30−30	+6 +6	−30−30	+4 ×
	1.2	8.8	6,000			−2	−6	−6	+10	−30−30				+5 +6	+6−30	+6 +6	+7−30	+4 +4
	1.0 (D)	9.0	5,000	−2−2	−2 −2	−2	−6	+6	+10	−10+30	+7−30	+14−30	+12−30	+6 ×	−30−30	+11+14	−30−30	+4 +4
	8	2	4,000						+10	−10−30	+7+12	× ×	+6+10	−30 ×	+8−30	−30×	+17−30	+4 +4
	6	4	3,000						+10	−10+30	+6+14	+ 7×	+10+10	−30−30	+8×	+6+12	+27+27	+6 ×
	4	6	2,000						−10	−10+30	+6−30	+17−30	+20−30	−30−30	+8−30	+27−30	−30−30	+6 +6
D	2	8	1,000						−10	−10−30	+10+14	+21×	−30−30	−30−30	−30−30	−30−30	−30−30	+6 ×
(1C+9H_2O)	1.8	8.2	900						−10	−10+30	+6×	−30×	+10+16	−30−30	−30−30	+25−30	−30−30	−30−30
	1.6	8.4	800						−10	−10+30	+14+22	+21−30	−30×	−30−30	−30−30	+20−30	+27−30	−30−30
	1.4	8.6	700						+10	−10−30	−30−30	+30×	−30×	−30−30	+8+10	+30−30	+27−30	+8×
	1.2	8.8	600						−10	−10+30	+27−27	−30−30	−30×	−30−30	+13−30	−30−30	−30−30	+6−30
	1.0 (E)	9.0	500	−2−2	−2 −2	−2	−6	−6	−10	−10−30	−30−30	−30−30	−30×	−30−30	−30−30	−30−30	+27+27	−30−30

| | | |

The length of the incubation period in relation to the decreasing number of spores used varied greatly with the different lots of spores, even with the duplicate inoculations of each lot. Table 5 gives the results of positive cultures obtained in relation to the period of incubation and the dilution of the spores. The coefficient of correlation (*14, p. 179*) for the positive cultures only, in relation to length of incubation and dilution of spores, was found to be 0.3558 ± 0.0440. While this does not show a strong correlation, it indicates that with the smaller numbers of spores there is a tendency for growth to take place with longer periods of incubation. However, when the cases of positive growth were correlated with the dilution and incubation time on the basis of the percentage of positive cultures to negative cultures for each observation period of incubation time, an insignificant negative correlation was obtained. Apparently there is a variable uncontrollable factor present, more obvious when spores are used from different lots of the organism, which makes it impossible to correlate the other factors closely. However, the data summarized in Table 6 indicate that, of the 120 cultures made with seedings of between 5,000,000,000 and 9,000,000 spores per seeding, 98.33 per cent showed growth at the end of 10 days' incubation, while 100 per cent (120 cultures) showed growth after 30 days' incubation. This is 56.87 per cent of the 211 total cultures showing growth after 30 days.

TABLE 5.—*Summary of the positive vegetative cultures of Bacillus larvae in relation to length of incubation period and with varying dilutions of the stock suspension*

Number of spores per cubic centimeter inoculated	Number of positive cultures after days of incubation																												Number of cultures		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Positive	Negative	Total
5,000,000,000	16																													16	0	16
500,000,000	16																													16	0	16
50,000,000	4	6	4	2																										16	0	16
40,000,000	4	3	6	3																										16	0	16
30,000,000	2	4	5	4	1																									16	0	16
20,000,000		6	5	2	1	2																								16	0	16
10,000,000		2	2	1	4	3	1						1																	14	0	14
9,000,000			4		4			1																		1				10	0	10
8,000,000			4		2					1					1															8	2	10
7,000,000			3		2																									5	4	9
6,000,000			2	1	4	1																								8	2	10
5,000,000			2		1	1				1	1		2																	8	7	15
4,000,000			2		1	1	1		1		1					1														8	4	12
3,000,000					3	1	1		2		1		1													2				11	2	13
2,000,000					3		1									1			1							1				7	9	16
1,000,000					1				1				1							1										4	10	14
900,000					1				1						1									1						4	10	14
800,000													1						1	1	1					1				5	10	15
700,000							2		1																	1			2	6	7	13
600,000					1							1														1				3	12	15
500,000																										2				2	13	15
400,000																														0	15	15
300,000																										1				1	15	16
200,000													1																	1	15	16
100,000																													1	1	15	16
90,000							1																							1	15	16
80,000							1																						1	2	14	16
70,000					1																					2				3	12	15
60,000							1																							1	15	16
50,000										1		1																		2	14	16
0–40,000																														0	123	123
Total positive cultures	42	21	30	13	30	9	9	1	6	3	3	2	7	0	2	2	0	0	2	2	1	0	0	1	0	12	0	0	4	211		
Total negative cultures	514	493	454	441	411	402	393	392	386	383	380	378	371	371	369	367	367	367	365	363	362	362	362	361	361	349	349	349	345		345	
Total cultures																																556

TABLE 6.—*Summary of positive vegetative cultures of Bacillus larvae grouped in relation to size of seedings and length of incubation period*

Number of spores per seeding	10 days' incubation				11 to 30 days' incubation				Final observations after 30 days' incubation								
	Number of positive cultures	Number of negative cultures	Percentage positive	Percentage negative	Number of positive cultures	Number of negative cultures	Percentage positive	Percentage negative	Total number of positive cultures	Total number of negative cultures	Total number of all cultures	Percentage positive	Percentage negative	Percentage positive of total of all positive cultures	Percentage negative of total of all negative cultures	Percentage positive of total of all cultures	Percentage negative of total of all cultures
5,000,000,000–9,000,000	118	2	98.33	1.66	2	0	1.67	0	120	0	120	100	0	56.87	0	21.58	0
8,000,000–500,000	48	123	28.07	71.93	31	92	18.12	53.80	79	92	171	46.20	53.80	37.44	26.66	14.21	16.55
400,000–50,000	4	138	2.82	97.18	8	130	5.63	91.55	12	130	142	8.45	91.55	5.69	37.68	2.16	23.38
40,000–0	0	123	0	100	0	123	0	100	0	123	123	0	100	0	35.65	0	22.12
Total	170	386	30.58	69.42	41	345	7.37	62.05	211	345	556	----------	----------	----------	----------	37.95	62.05

Of the 171 cultures made with seedings between 8,000,000 and 500,000 spores per seeding, 48, or 28.07 per cent, showed growth at the end of 10 days' incubation, while 79, or 46.20 per cent, showed growth after 30 days' incubation. The latter number is 37.44 per cent of the 211 total cultures showing growth after 30 days' incubation.

Of the 142 cultures made with seedings between 400,000 and 50,000 spores per seeding, only 4, or 2.82 per cent, showed growth at the end of 10 days' incubation, while 12, or 8.45 per cent, showed growth after 30 days' incubation. The latter figure is 5.69 per cent of the 211 cultures showing growth after 30 days' incubation.

Of the 123 cultures made with seedings of 40,000 or fewer spores per seeding, no growth was obtained after 30 days' incubation.

Of the 556 cultures made with all seedings, 30.58 per cent showed growth at the end of 10 days' incubation and 69.42 per cent showed no growth. The 170 positive cultures after 10 days' incubation is 80.57 per cent (not shown in Table 6) of the 211 total positive cultures obtained. In the interval between the 10 and 30 day incubation periods, 19.43 per cent (not shown in Table 6) of the 211 total positive cultures, or another 7.37 per cent of all cultures made, showed growth, making a total of only 37.95 per cent of all cultures which showed growth at the end of 30 days' incubation, with 62.05 per cent still showing no growth.

The initial growth phases as described by Buchanan (*3; 13, Ch. V*) are clearly more marked with spores than with simple vegetative organisms, since there is a varying length of time necessary for spores to germinate and start growing after implantation in a suitable medium. In the light of observations on other spore-forming organisms, it is probable that this factor, which seems to cause variations in the germination time of *Bacillus larvae* even within a lot from a single source, is what has been termed "dormancy." Burke (*5, p. 283*), working with *Clostridium botulinum*, found:

> The individual (unheated) spores in a given culture of *Cl. botulinum* vary greatly in the time required for germination under optimum growth conditions. The majority germinate relatively quickly, but a few lie dormant for a longer time. One hundred and forty-four days is the maximum period of dormancy recorded here * * *.

Burke states:

> The primary factors which cause the spore to lie dormant for long periods of time under optimum growth conditions are believed to be inherent in the spore itself. It is thought that relative permeability of the spore wall is one of the factors. Environmental conditions may secondarily modify the period of dormancy.

Burke, Sprague, and Barnes (*6, p. 560*) observed the same phenomenon with such non spore-bearing bacteria as *Bacillus coli* (=*Escherichia coli*). They found that spores of *B. subtilis* remained dormant 39 days and those of *B. megatherium* 90 days, although a large majority developed in 4 or 5 days. They believe:

> Dormancy must be considered a factor in infection. It reduces the chances of infection by reducing the number of organisms that would otherwise start to grow at one time. Since the cells begin to multiply at different times, the body has an opportunity to initiate defensive reactions before all the cells develop. If dormant for a sufficient period, the organisms will be excluded from the body before development takes place.

Swann (*28*) has observed that there is a variation in the germination time of anthrax spores, depending on the age and condition of the spores.

Morrison and Rettger (*24, p. 339*) recently stated—

> Because of the marked variability of germination, depending upon the stimuli supplied in the environment, the deduction is made that bacterial spores in the process of germination are vitally active bodies having requirements for metabolic function which are the same as or more exacting and specific than those of the vegetative cells.
>
> Experimental evidence is presented to show that the dormancy of aerobic bacterial spores is largely, if not entirely, determined by conditions in the environment of the spores, and that these factors must be taken into consideration, perhaps specifically for each species, before so-called "inherent" or "normal" dormancy of bacterial spores can be established.

This phase of the work with *Bacillus larvae* is being repeated with the organism obtained from a single source in an effort to determine the importance of this variable factor of dormancy.

SPORES OF BACILLUS LARVAE IN COMMERCIAL HONEY

A few instances have been reported in the bee journals, such as that by Merrill (*22*), in which American foulbrood has developed as a result of bees having access to cans of infected honey that have been carelessly thrown out. Without doubt in some cases honey has been allowed to get on the market from infected colonies through negligence of the beekeepers and without being diluted by mixing or blending with honey from disease-free apiaries. On the other hand, Fracker (*10, p. 379–380*) has shown, by a study of disease-inspection statistics for Wisconsin:

> 1. In Wisconsin the introduction of this disease into the State and into many individual localities is definitely known to have been in specific importations of bees and equipment.
> 2. Cases of infection in which the source appears to be infected honey in the channels of trade are comparatively rare.
> 3. Even near such a large center as Milwaukee the infection percentage is greatest in localities of active movement, such as greenhouse areas, and is relatively low within the city itself.
> 4. Towns and cities of from 3,000 to 40,000 which have been natural markets for infected honey from near-by counties, have remained for years free from disease either until the present or until infected bees and equipment were introduced.
> 5. No new centers of infection are known to have been started since the policy of limiting movement of bees and equipment was begun in 1919.
> 6. These observations appear to be confirmed by conditions in the South, in spite of the fact that the period of active flight of the bee tends to continue through the peak of honey distribution.

Furthermore, F. L. Thomas, State entomologist of Texas, in an unpublished manuscript states:

> The largest of the estimates with reference to the quantity of honey that is brought into Texas in a year is 19 carloads. Most of this honey is produced in California, Colorado, New Mexico, Utah, and Wyoming. * * *
>
> If 19 carloads of foulbrood-infected honey are distributed annually in this State, it seems reasonable to suppose that our inspectors would have a hard time to keep this disease within bounds. In fact, I would expect to find that the inspectors would be gradually losing ground in their attempts to eradicate this menace. A large share of the honey which is imported is sold in west and northwest Texas where practically no bees are kept. The amount which is distributed in the beekeeping territory of the State is evidently less dangerous than is commonly supposed. The following facts, I think, will prove this statement.
>
> During the period September 1, 1920, to August 31, 1926, the inspection work has been carried into 100 counties. Fifty-six counties were found to be free

from contagious or infectious diseases of bees, but in the other 44 counties American foulbrood has been present.

An average of 668 beekeepers have been visited each year and 38,661 colonies examined with the result that an average of 430 colonies, or 1.11 per cent, have been found to be diseased.

American foulbrood is found now in only 23 counties, 21 of the 44 counties having been cleaned up. In 12 of the counties where disease occurs, only 30 colonies were found to be infected out of 7,642 examined—less than 0.4 of 1 per cent. Six counties had one diseased colony each.

About 40 per cent of the beekeepers and 60 per cent of the colonies are reinspected from year to year; the remainder, being free of disease and considered out of danger, are dropped and "new territory" is taken over and examined for presence of foulbrood. By "new territory" is meant beekeepers and their colonies visited and inspected for the first time. An average of 228 diseased colonies are discovered each year in "new territory." This is 1.6 per cent of the total number of colonies examined in this territory.

The reinspection which has been made in the counties where disease has been present shows that there have been both gains and losses. But a net gain has resulted which has averaged 21 beekeepers and 368 colonies freed from American foulbrood and quarantine annually.

From these facts it is easily seen that definite and really rapid progress in eradicating the disease is being made. Rarely do our inspectors find new outbreaks of disease that can not be traced to careless beekeeping methods, bees robbing infected and weakened colonies, or to the use of old and infected equipment.

It is not my intention to imply that honey is not a carrier of American foulbrood. The above evidence simply indicates that the honey which has been imported into Texas has not been as dangerous a source of disease to bees as is sometimes thought.

Practically no work has been reported on the microbiology of honey other than that in connection with the spoilage of honey through fermentation by yeasts (*19*, *21*), and no work appears to have been done on the *Bacillus larvae* spore content of commercial honey. In 1925 the writer undertook to devise a method for demonstrating, at least qualitatively, the presence or absence of spores of *B. larvae* in honey and their significance in relation to the results of the spore-feeding experiments. Difficulties were encountered in obtaining cultures of *B. larvae* from honey. It was impossible to obtain vegetative growth of this organism, even when a considerable number of spores had previously been added to honey, because of the difficulty of eliminating contaminating organisms that developed rapidly in the honey, completely overgrowing any possible vegetative growth of *B. larvae* before it could get well started. Therefore, methods of concentrating the spores from the honey and of identifying them by means of microscopic examination were attempted. Because spores of *B. larvae* have a characteristic appearance in stained smears (*20*, *p. 9*), it was assumed that this method might give at least tentative evidence.

METHODS OF PROCEDURE

The first method attempted was the filtration of honey diluted with water through a membrane of ether-alcohol collodion or through filter paper impregnated with an acetic acid solution of collodion (*9*). Apparatus was devised in which both suction and pressure were tried in this filtering process. Stained smears were made of the sediment retained on the surface of the filter. In several cases spores of *Bacillus larvae* were observed in stained smears of the sediment filtered out of honey known to have a large spore content. However, with honey containing fewer spores it was found impossible to concentrate them on a small enough area of filter in sufficient

numbers to recover and identify them under the microscope. Even with a comparatively large filtering surface, the process was so slow that the diluted honey would frequently start to ferment before it had all passed through the filter. A filter of smaller area would become clogged, preventing the passage of a sufficient quantity of honey.

Several unsuccessful attempts were made to recover spores of *Bacillus larvae* from honey by centrifuging samples diluted with an equal quantity of water. After considerable experimentation with honey of known spore content, it was found that it was necessary to dilute the honey to a much greater extent—1 part to at least 9 of water—in order to throw the spores down with the sediment. Apparently the specific gravity of these spores is so low that on centrifuging they remain in suspension in only slightly diluted honey.

The procedure finally used for demonstrating the presence of spores of *Bacillus larvae* in honey is as follows: Five c c of warmed honey is thoroughly mixed with 45 c c of distilled water in a 50 c c cone-shaped centrifuge tube made of heat-resistant glass. Duplicate quantities of each sample of honey are made up for examination. The diluted honey is then centrifuged at 2,000 revolutions per minute for one-half hour. Because of the difficulty of obtaining a satisfactory stained smear from the sediment thrown down in the presence of the sugars of the honey solution, all but 2 c c of the solution in each centrifuge tube is drawn off by means of a 50 c c pipette. Another 45 c c of distilled water is added, the sediment is thoroughly shaken up in the water, and the tubes are centrifuged again for 20 minutes. After all but 2 c c or less of the wash water has been removed, 0.01 c c of the sediment is removed by means of a capillary pipette and smeared on a cover glass over a surface of 1 cm^2, a small loopful of carbol fuchsin being mixed with the material before it is allowed to dry. After drying by gentle heat, the cover glass is mounted on a slide by means of a drop of distilled water and the smear is examined with an oil-immersion objective. Spores of *B. larvae* are identified by their size and shape in conjunction with their distinctive habit of breaking loose from the stained mass of the smear and of showing a delicate Brownian movement in the thin film of water between the two pieces of glass. In a few samples only one or two spores were seen in numerous fields examined or the spores did not have the typical appearance of spores of *B. larvae*. In such cases another test, in which twice as much honey was used, was made from the sample.

OBSERVATIONS

One hundred and ninety-one samples of honey were examined by this method. (Table 7.) Of these, 187 were regular commercial samples purchased in the open market and 2 were from the experimental apiary at Laramie. The other two were miscellaneous samples, one of which was obtained from a brood comb from a diseased colony and the other from a cappings melter which had been used with combs from an infected apiary.

TABLE 7.—*Results of the examination of samples of honey for the presence of spores of Bacillus larvae*

Source	Samples tested	Samples showing positive presence of spores resembling *Bacillus larvae*	Samples showing no evidence of spores [a]
Commercial samples from 30 States	187	15	172
Experimental	2		2
Miscellaneous	2	2	
Total	191	17	174

[a] 29 of these samples were doubtful on the first examination, but repeated examinations gave negative results in each case.

Of the 187 samples of commercial honey obtained from 30 different States or Territories, 15, or 8 per cent, showed the presence of a sufficient number of spores resembling spores of *Bacillus larvae* to be designated as positive. In 29 of the commercial samples, or 15.5 per cent, one or two doubtful spores were seen in each case, but on repeated examinations none of these samples could be considered positive. Two of the four miscellaneous samples from infected sources were also found to contain spores of *B. larvae*.

Five of the samples showing the presence of spores of *Bacillus larvae* were fed to healthy 5-frame colonies during the summer of 1930. These samples consisted of from a pint to a quart of honey. No evidence of American foulbrood appeared in any of the five colonies during the entire brood-rearing season.

In order to determine the approximate number of spores in the samples of honey in which the presence of *Bacillus larvae* was demonstrated, a series of dilutions of spores was prepared as described for the work with cultures. A stained smear was made of 0.01 c c of each dilution spread over a 1-cm² surface of cover glass mounted with water and examined with the oil-immersion objective. By this means a definitely recognizable number of spores could be found down to the dilution of 2,000,000 spores per cubic centimeter, with a few single spores seen in occasional fields down to the dilution of 500,000 spores per cubic centimeter. (Table 8.) Then 1 c c of each dilution was added to 5 c c of distilled water in 15 c c centrifuge tubes and centrifuged at 2,000 revolutions per minute for 20 minutes. A stained smear made from 0.01 c c of each sediment showed a definitely recognizable number of spores down to the 5,000-spore dilution, with one or two doubtful spores in several fields from the 500-spore dilution. The sample containing the 50,000-spore dilution, which would be comparable to the sugar sirup containing the minimum number of spores per cubic centimeter fed to colonies in the spore-feeding experiments that produced infection, showed a great many more spores in each field examined by this method than did the sample of commercial honey that showed the greatest number of spores. Therefore, until a better quantitative method is devised, it seems reasonable to believe, from the indications of the preliminary work on this problem, that, even though the presence of a few spores of *B. larvae* may be

demonstrated in 5 c c quantities from a comparatively small percentage of samples of commercial honey, the numbers are far below the minimum necessary to produce infection when such honey is used in healthy colonies of bees. Before definite conclusions can be drawn, it will be desirable to examine many more samples of commercial honey and to feed to healthy colonies samples of honey in which the presence of spores has been demonstrated.

TABLE 8.—*Microscopic examination of dilutions for spores of Bacillus larvae* [a]

Number of spores per cubic centimeter in each dilution	Direct examination of 0.01 cubic centimeter	Examination of sediment after centrifuging 1 cubic centimeter	Number of spores per cubic centimeter in each dilution	Direct examination of 0.01 cubic centimeter	Examination of sediment after centrifuging 1 cubic centimeter	Number of spores per cubic centimeter in each dilution	Direct examination of 0.01 cubic centimeter	Examination of sediment after centrifuging 1 cubic centimeter
5,000,000,000	+	+	10,000,000	+	+	100,000	−	+
4,000,000,000	+	+	9,000,000	+	+	90,000	------	+
3,000,000,000	+	+	8,000,000	+	+	80,000	------	+
2,000,000,000	+	+	7,000,000	+	+	70,000	------	+
1,000,000,000	+	+	6,000,000	+	+	60,000	------	+
500,000,000	+	+	5,000,000	+	+	50,000	−	+
400,000,000	+	+	4,000,000	+	+	40,000	------	+
300,000,000	+	+	3,000,000	+	+	30,000	------	+
200,000,000	+	+	2,000,000	+	+	20,000	------	+
100,000,000	+	+	1,000,000	±?	+	10,000	------	+
90,000,000	+	+	900,000	------	+	5,000	−	+
80,000,000	+	+	800,000	------	+	4,000	------	------
70,000,000	+	+	700,000	------	+	3,000	------	------
60,000,000	+	+	600,000	------	+	2,000	------	------
50,000,000	+	+	500,000	±?	+	1,000	------	------
40,000,000	+	+	400,000	−	+	500	−	±
30,000,000	+	+	300,000	−	+	50	−	−?
20,000,000	+	+	200,000	−	+	5	−	−

[a] + indicates that spores were found; − indicates that spores were not found, by microscopic examination; ± indicates that the result was doubtful; ±? indicates that the positive was more doubtful than the negative; −? indicates that the absence of spores was not definite.

SUMMARY AND CONCLUSIONS

As a result of five years' study it has been found that, in order to produce American foulbrood infection in a healthy colony of bees, the sugar sirup used for inoculation must contain a certain initial number of spores of *Bacillus larvae*. Seventy-three colonies were inoculated during this time with numbers of spores ranging from approximately 5,000,000,000 to 100,000 per colony; 30 of these colonies receiving 50,000,000 spores or less. Of these 30 colonies, 2 out of 11 receiving 50,000,000 spores showed infection, but no colony receiving less than that number of spores developed disease. Therefore, the minimum infectious dose of *B. larvae* for a colony of bees seems to be approximately 50,000,000 spores in 1 liter of sugar sirup.

Preliminary experiments in which individual bee larvae were given known numbers of spores of *Bacillus larvae* in 0.01 c c quantities of sugar sirup show that infection can be produced by this method, but with considerable difficulty. From 50 to 100 larvae were inoculated with each dilution of spores, ranging in number from approximately 50,000,000 spores to, theoretically, 1 spore per larva. The minimum infectious dose was found to be 10,000,000 spores per larva fed in 0.01 c c of sugar sirup. These results indicate that the

minimum dose of spores of *B. larvae* that will produce American foulbrood infection must be large.

The germination of spores of *Bacillus larvae* and vegetative growth on a suitable artificial culture medium resulting from the inoculation of 556 culture tubes with seedings varying from approximately 50,000,000,000 to 500 spores per culture also shows that a certain minimum initial number of spores in the inoculum is necessary in order to produce growth. This minimum number of spores producing vegetative growth on a medium consisting of yeast-carrot extract, egg-yolk suspension, and agar was found to be approximately 50,000 in 1 c c of suspension inoculated.

The production of nitrite in this medium by the vegetative growth of *Bacillus larvae* serves as a fairly delicate and reliable indicator of such growth.

There was a tendency for the seedings containing the smaller numbers of spores of *Bacillus larvae* to require a longer period of incubation than the larger seedings in order to produce vegetative growth. However, there was a considerable variation in the germination time of many of the seedings of spores, in one case a seeding of 9,000,000 spores requiring 27 days' incubation to produce growth and another of 70,000 spores requiring only 6 days. This variation, thought to be due to the variable character known as dormancy in bacterial spores, prevented more than a slight correlation.

In the group of cultures comprising seedings between 5,000,000,000 and 9,000,000 spores, only 1.67 per cent required more than 10 days' incubation to produce vegetative growth, 100 per cent having shown growth after 30 days. In the group of cultures comprising seedings between 8,000,000 and 500,000 spores, 71.93 per cent required more than 10 days' incubation, while 53.81 per cent showed no growth at the end of 30 days' incubation. In the group of cultures comprising seedings between 400,000 and 50,000 spores, 97.18 per cent required more than 10 days' incubation, while 91.55 per cent of the group showed no growth at the end of 30 days. Below 50,000 spores no growth was obtained. In other words, below a seeding of 9,000,000 spores an increasing number of the smaller spore seedings required a longer period of incubation. About 80 per cent of all the positive cultures were obtained during the first 10 days of incubation, although this was approximately only 30 per cent of all the cultures made; at the end of 30 days' incubation only about 38 per cent of all the cultures had shown any growth.

It was found possible to demonstrate the presence of spores of *Bacillus larvae* in 15 out of 187, or in 8 per cent, of the samples of commercial honey examined by means of the centrifuge and the microscope. The preliminary results indicate that, even though spores of *B. larvae* may be demonstrated in a certain percentage of samples of commercial honey, in most instances they are probably present in such small numbers as to be less than the minimum number, 50,000,000 per liter, found to be capable of producing disease, and therefore are ineffective in the spread of American foulbrood.

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HETEROTHALLISM AND HYBRIDIZATION IN SPHACELOTHECA SORGHI AND S. CRUENTA [1]

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INTRODUCTION

It has been shown by a number of investigators that certain of the smut fungi are heterothallic. This means that in such fungi the fusion of two hyphal or monosporidial lines of opposite sex is necessary before normal infection and the subsequent production of chlamydospores can take place. Kniep (*14*)[3] in 1919, working with *Ustilago violaceae* (Pers.) Fckl., was the first to show that fusion takes place only between certain sporidia. Evidence of heterothallism in other smut fungi was later reported by Bauch (*1*), Stakman and Christensen (*16*), Dickinson (*5*), Hanna and Popp (*12*), and Flor (*9*). Fusion between monosporidial lines of opposite sex of different species of smut fungi has been observed. Kniep (*15*), working with several species of Ustilago, observed fusion between monosporidial lines of different species. Dickinson (*6*) was unable to obtain infection of oat seedlings with monosporidial lines of *U. levis* (Kell. and Sw.) Magn. and *U. hordei* (Pers.) Kell. and Sw., but when sporidia of one sex of *U. hordei* were combined with those of opposite sex of *U. levis*, infection resulted. Apparently his study was not carried far enough to determine whether hybrid chlamydospores could be produced as a result of the cross. Hanna and Popp (*12*) and Holton (*13*) obtained hybrid chlamydospores as a result of interspecific crosses made between *U. levis* and *U. avenae*. The hybrid chlamydospores were found to be echinulate, while chlamydospores from inbred lines of each species had markings characteristic of the respective species. Flor (*9*) recently found *Tilletia levis* Kühn and *T. tritici* (Bjerk.) Wint. to be heterothallic. Hybrid chlamydospores were obtained as a result of crosses between physiologic forms of a single species and of crosses between the two species. The chlamydospores produced by the species cross were identical in appearance with those of *T. levis*.

The fact that certain of the smut fungi have been found to be heterothallic and that hybrid chlamydospores may be produced as a result

[1] Received for publication Mar. 25, 1932; issued September, 1932.

[2] The writer wishes to express his appreciation to A. G. Johnson, principal pathologist, Division of Cereal Crops and Diseases, for assistance in the preparation of the manuscript. He is indebted also to Prof. L. E. Melchers, of the Kansas State Agricultural College, for chlamydospore material of the three physiologic forms of *Sphacelotheca sorghi* and the two collections of *S. cruenta*.

[3] Reference is made by number (italic) to Literature Cited, p. 295.

of intraspecific and interspecific crosses suggests strongly that new physiologic forms may arise as a result of hybridization. In a study of the origin of pathogenically different strains of *Sphacelotheca sorghi* (Lk.) Clint., the present investigations were made to determine whether *S. sorghi* and *S. cruenta* (Kühn) Potter are heterothallic and to what extent hybridization may take place between physiologic forms and between the two species.

MATERIALS AND METHODS

Single sporidial lines used in these tests were isolated from chlamydospores of three pathogenically distinct physiologic forms of *Sphacelotheca sorghi*, namely, the common, milo, and feterita strains as identified by Tisdale, Melchers, and Clemmer (*17*), and from two collections of *S. cruenta*.[4] The method used in isolating single sporidia was similar to that described by Dickinson (*4*) and Hanna (*10*), except that the isolating needle was attached to a Chambers micromanipulator. Single chlamydospores taken from a single sorus were placed on hanging drops of 2 per cent potato-dextrose agar, and when they had germinated the sporidia were removed from the promycelia and propagated separately. Subcultures of these lines, to be used as inoculum, were prepared by growing each line separately in a 4 per cent carrot extract. The cultures were allowed to develop three weeks before being used to inoculate sorghum plants.

Reed kafir sorghum (C. I.[5] 628), which is susceptible to all known collections of both *Sphacelotheca sorghi* and *S. cruenta*, was used in all the experiments. When the plants were 5 weeks old they were inoculated with the sporidial suspensions. By means of a hypodermic syringe the inoculum was injected into the plants as near the growing point as possible. When a combination of two monosporidial lines was used the two cultures were mixed just before the inoculation. Controls were inoculated in the same manner with sterile carrot extract. The plants were grown singly in pots in the greenhouse until six days after they were inoculated, and then transplanted to the field plots at the Arlington Experiment Farm, Rosslyn, Va. (near Washington, D. C.). Ten plants were used for each inoculation. In some of the tests, as indicated later, the plants were grown to maturity in the greenhouse.

The common, milo, and feterita strains of *Sphacelotheca sorghi* are referred to in this paper as forms S1, S2, and S3, respectively, and collections of *S. cruenta* as C1 and C2. Where different chlamydospores of a particular form or collection were used they are distinguished by a letter. In most cases a record was kept of the position on the promycelium occupied by each sporidium, and for convenience the sporidia are numbered 1, 2, 3, and 4, from the tip of the promycelium to the chlamydospore. Thus, S1B3 would refer to a sporidium isolated from the third segment of the promycelium from a particular chlamydospore B of form 1, *S. sorghi*.

[4] The collections of *Sphacelotheca cruenta* referred to in this paper as C1 and C2 were received from Professor Melchers as Reed's and Ficke's collections, respectively.

[5] C. I. denotes accession number of Division of Cereal Crops and Diseases, formerly Office of Cereal Investigations.

HETEROTHALLISM

SPHACELOTHECA SORGHI

Each of the four first-formed sporidia from each of three chlamydospores of *Sphacelotheca sorghi*, form 1, was isolated and cultured as previously described. Subcultures of these monosporidial lines, alone and in all possible paired combinations for each chlamydospore, were then used to inoculate sorghum plants. The results of these inoculations are presented in Figure 1. The plus sign (+) indicates that the inoculated plants developed smutty heads, and the minus sign (−) indicates that no smut developed.

It is evident from the results obtained that *Sphacelotheca sorghi* is heterothallic, since none of the 12 monosporidial lines when injected alone caused infection, whereas plants inoculated with certain paired combinations or sex groups developed smutty heads. Bauch (*2*) found that *Ustilago longissima* (Sow.) Tul. might have sporidia belonging to many sexual groups. Hanna (*11*) and Christensen (*3*) showed that at least four sexual groups might be present in sporidia

	1	2	3	4
1	−	+	−	+
2	+	−	+	−
3	−	+	−	+
4	+	−	+	−

S1A

	1	2	3	4
1	−	+	+	+
2	+	−	−	−
3	+	−	−	−
4	+	−	−	−

S1B

	1	2	3	4
1	−	+	+	−
2	+	−	−	+
3	+	−	−	+
4	−	+	+	−

S1C

FIGURE 1.—Results of inoculating sorghum plants with monosporidial lines of *Sphacelotheca sorghi* used singly and in pairs, from each of the chlamydospores S1A, S1B, and S1C

of *Ustilago zeae* (Beckm.) Ung. In the present investigations with *S. sorghi*, sporidia belonging to only two sexual groups have been isolated. However, with reference to sex the sporidia were arranged on the promycelium in three different positions. From chlamydospore S1A, sporidia 1 and 3 were of the opposite sex from that of sporidia 2 and 4; while from chlamydospore S1C, sporidia 1 and 4 were of the opposite sex from that of sporidia 2 and 3. Two sexual groups were present in sporidia from chlamydospore S1B, but they were not in pairs, sporidium 1 being of the opposite sex from that of sporidia 2, 3, and 4.

Christensen (*3*), working with *Ustilago zeae*, found that when the monosporidial lines isolated from a promycelium are paired in all possible combinations they may fall into two sexual groups, but that when paired with sporidia from another promycelium they frequently belong to three or four distinct sexual groups. In the present investigation all possible paired combinations were made between the four monosporidial lines from chlamydospores S1A and S1B. The

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results of these matings (fig. 2) indicate that the sex groups reacted normally and that no evidence was obtained of complete interfertility or intersterility. In greenhouse experiments similar results were obtained from several matings between sporidia of known sex of physiologic forms S1, S2, and S3.

		S1A				S1B			
		1	2	3	4	1	2	3	4
S1A	1	−	+	−	+	−	+	+	+
	2	+	−	+	−	+	−	−	−
	3	−	+	−	+	−	+	+	+
	4	+	−	+	−	+	−	−	−
S1B	1	−	+	−	+	−	+	+	+
	2	+	−	+	−	+	−	−	−
	3	+	−	+	−	+	−	−	−
	4	+	−	+	−	+	−	−	−

FIGURE 2.—Results of inoculating sorghum plants with four monosporidial lines of *Sphacelotheca sorghi* from chlamydospore S1A paired with the four monosporidial lines from chlamydospore S1B

SPHACELOTHECA CRUENTA

Each of the four first-formed sporidia from each of three chlamydospores of collection C1 of *Sphacelotheca cruenta* was isolated, cultured, and used in inoculations. The results of inoculating sorghum plants with these monosporidial lines alone and in paired combinations for each chlamydospore are recorded in Figure 3.

	1	2	3	4
1	−	+	+	−
2	+	−	−	+
3	+	−	−	+
4	−	+	+	−

C1A

	1	2	3	4
1	−	−	−	+
2	−	−	−	+
3	−	−	−	+
4	+	+	+	−

C1B

	1	2	3	4
1	−	+	+	+
2	+	−	−	−
3	+	−	−	−
4	+	−	−	−

C1C

FIGURE 3.—Results of inoculating sorghum plants with monosporidial lines of *Sphacelotheca cruenta* used singly and in pairs, from each of the chlamydospores C1A, C1B, and C1C

These data indicate that *Sphacelotheca cruenta* is heterothallic and that the sporidia from each promycelium belong to two sexual groups. The two groups from chlamydospore C1A were so arranged that sporidia 1 and 4 were of the opposite sex from that of sporidia 2 and 3. Chlamydospores C1B and C1C each produced three sporidia of one

sexual group and one of the opposite group. However, the arrangement differed in that the sporidia of C1B were grouped so that sporidia 1, 2, and 3 were of the opposite sex from that of sporidium 4 and those of C1C so that sporidium 1 was of the opposite sex from that of sporidia 2, 3, and 4.

A test was also made with *Sphacelotheca cruenta* to determine the sex relationship between sporidia of two different chlamydospores. The sporidia of chlamydospore C1A were paired in all possible combinations with those of chlamydospore C1B, and the results obtained are recorded in Figure 4. It is evident from these data that the sex groups reacted normally, as they did in the outbred lines of *S. sorghi*, and also that there is no indication of complete interfertility or intersterility.

From the evidence just presented it appears that in both *Sphacelotheca sorghi* and *S. cruenta* the factors determining sex may segre-

		C1A				C1B			
		1	2	3	4	1	2	3	4
C1A	1	−	+	+	−	+	+	+	−
	2	+	−	−	+	−	−	−	+
	3	+	−	−	+	−	−	−	+
	4	−	+	+	−	+	+	+	−
C1B	1	+	−	−	+	−	−	−	+
	2	+	−	−	+	−	−	−	+
	3	+	−	−	+	−	−	−	+
	4	−	+	+	−	+	+	+	−

FIGURE 4.—Results of inoculating sorghum plants with four monosporidial lines of *Sphacelotheca cruenta* from chlamydospore C1A paired with the four monosporidial lines from chlamydospore C1B

gate on a 2:2 or 1:3 basis. The sex factors of chlamydospores S1A, S1C, and C1A all segregated on a 2:2 basis, and from the arrangement of the sex groups on the promycelium it is evident that reduction of factors for sex took place in the second division of the fusion nucleus. Factors determining sex in chlamydospores S1B, C1B, and C1C segregated on a 1:3 basis. Segregation in this ratio is more difficult to explain. It may have taken place as a result of segregation in the third division of the nucleus or of some abnormal type of segregation. Hanna (*11*) and Christensen (*3*), working with *Ustilago zeae*, and Dickinson (*7*), working with *U. levis*, found similar segregation ratios for sex. These investigators found also that the segregation of sex factors may be independent of the segregation of factors for cultural characteristics. In the present investigation cultural comparisons of the four monosporidial lines from each of the three chlamydospores of *S. sorghi* and of *S. cruenta* were made on 2.5 per cent potato-dextrose

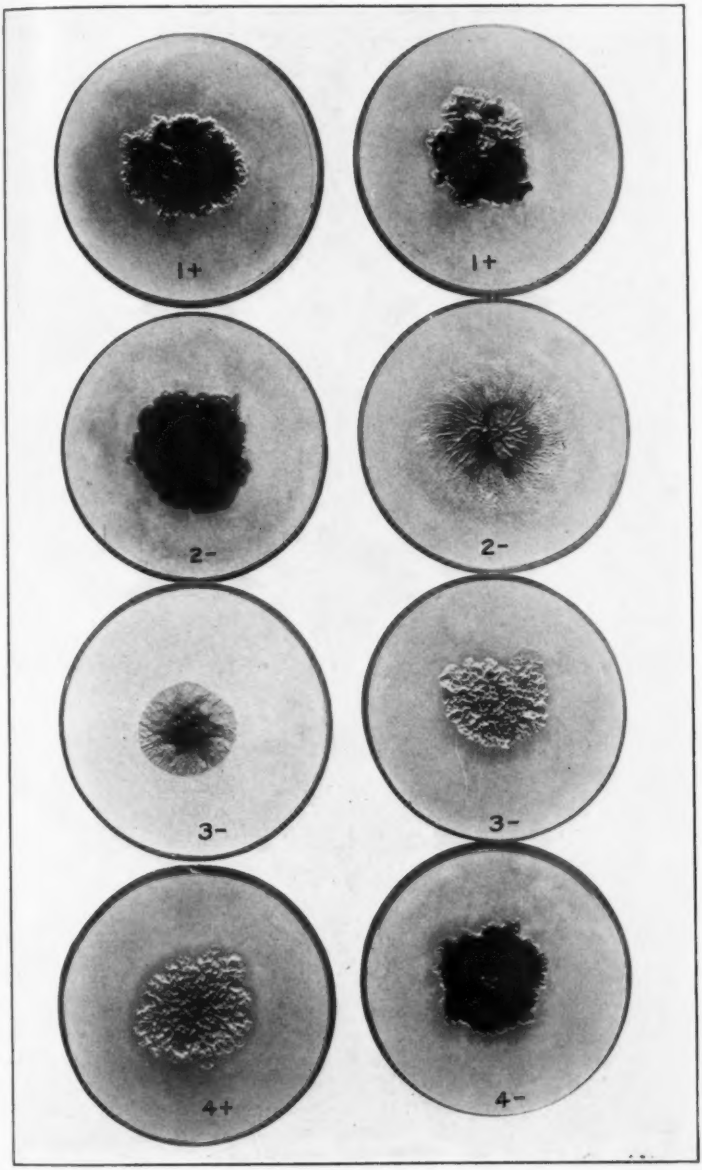
agar. The striking differences in the cultural characteristics of monosporidial lines from chlamydospores S1B and S1C (*S. sorghi*) may be seen in Plate 1. Similar differences in lines from chlamydospore C1C (*S. cruenta*) may be seen in Plate 2. It was evident from these tests that the factors determining sex in sporidia of both *S. sorghi* and *S. cruenta* segregated independently of those determining cultural characteristics. For example, the segregation ratio of factors for sex in chlamydospores S1A, S1B, and S1C of *S. sorghi* were 2:2, 1:3, and 2:2, respectively, while the factors determining color of the cultures of monosporidial lines from all these chlamydospores segregated on a 1:1:1:1 basis. In chlamydospores C1A, C1B, and C1C of *S. cruenta* the sex segregation ratios were 2:2, 1:3, and 1:3, respectively, as compared with the segregation ratios for color of colonies of 4:0 from chlamydospores C1A and 1:1:1:1 in lines from chlamydospores C1B and C1C. In these tests it was evident also that the factors determining other cultural characteristics such as topography, surface, consistency, rate of growth, and margin of colony segregated independently of those determining sex.

The ability of paired lines to produce chlamydospores in the plant has been considered in this paper as the criterion of sex compatibility. If two lines did not produce chlamydospores they were considered to be of the same sex. Some evidence was obtained that sex compatibility or incompatibility could be detected within a few days after the plants had been inoculated with the paired lines. It was found that on the leaves of plants inoculated with certain paired monosporidial lines of *Sphacelotheca sorghi* or *S. cruenta* small but distinct chlorotic areas developed in four to six days after inoculation, whereas the leaves of plants inoculated with certain other matings or with a monosporidial line remained normal. (Pl. 3.)

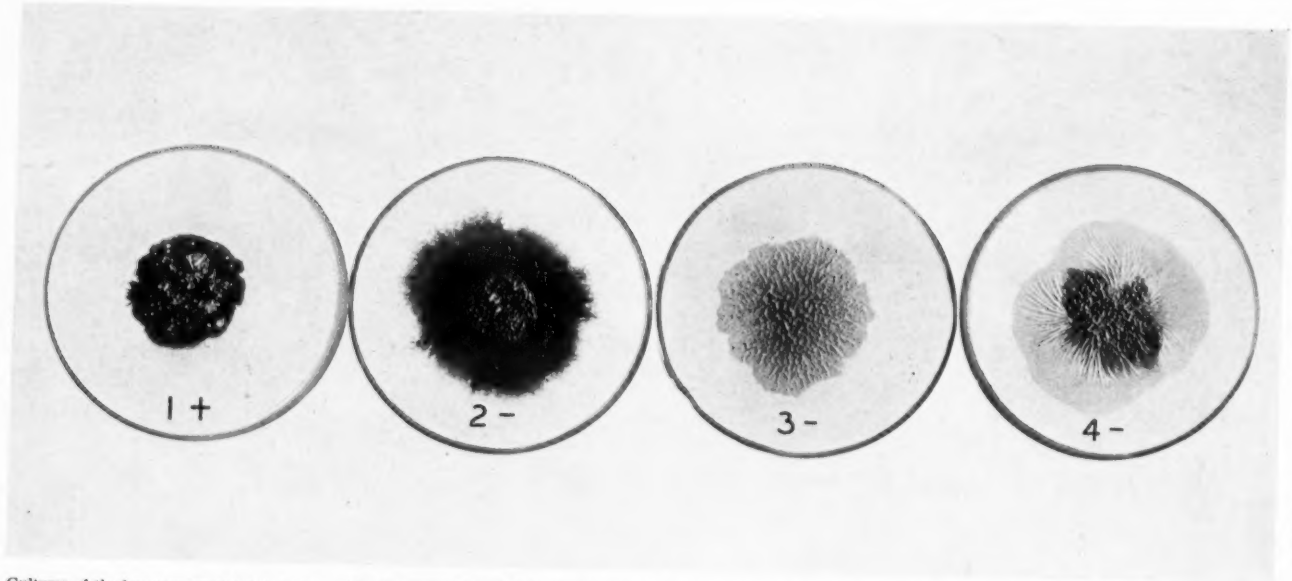
Under the conditions of these experiments nearly all the chlorotic spots disappeared within a week or 10 days after they first developed. However, on a few leaves of plants that had been inoculated with paired lines of *Sphacelotheca sorghi* the spots did not disappear and galls about the size of a beet seed developed, within which mature chlamydospores were formed. These chlamydospores were found to germinate normally. When all the plants had matured it was found that smutty heads developed only on those plants on whose leaves chlorotic spots had previously formed. In only one case did a paired line that caused flecking of the leaves fail to produce smut in the head. From these data it appears that the formation of the chlorotic spots is an early indication of sex compatibility, the factor or factors for which are closely associated if not linked with those for pathogenicity.

HYBRIDIZATION

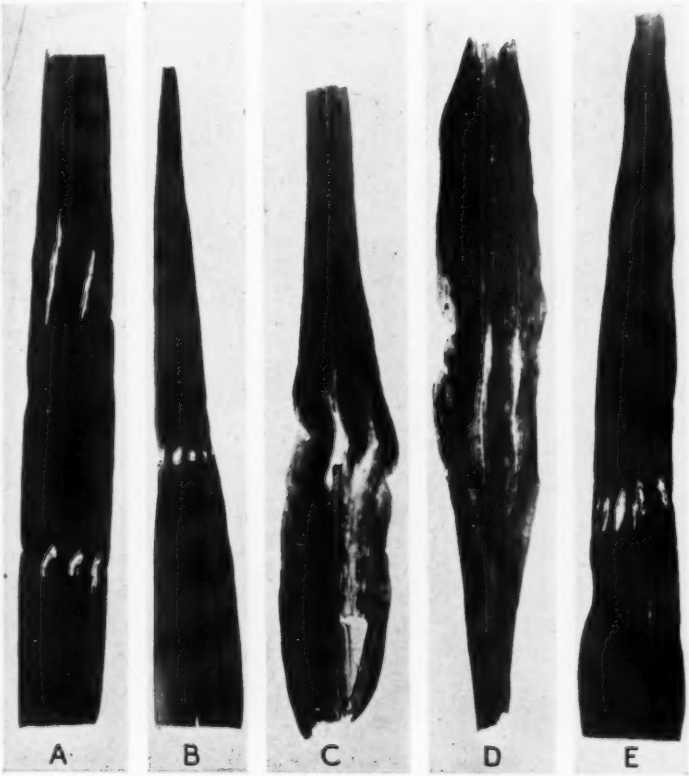
The four monosporidial lines isolated from chlamydospore S1A (*Sphacelotheca sorghi*) were paired in all possible combinations with those from chlamydospore C1A (*S. cruenta*). Reed kafir plants were inoculated with these combinations, and the results are recorded in Figure 5. It is clear from these data that *S. sorghi* and *S. cruenta* hybridize readily, as shown by the production of chlamydospores in the host. Furthermore, the sex groups as identified in the experiment on heterothallism reacted normally in the interspecific crosses. Similar results were obtained in greenhouse tests with a monosporidial



Cultures of monosporidial lines from two chlamydospores (S1B and S1C) of *Sphacelotheca sorghi* on potato-dextrose agar. The four cultures at left are from chlamydospore S1C; those at right are from chlamydospore S1B. The cultures are numbered 1, 2, 3, and 4, according to the position of the sporidia, from the tip of the promycelium to the chlamydospore. The sex is indicated by a plus (+) or minus (−) sign, according to the pairing reactions shown in Figure 1



Cultures of the four monosporidial lines from chlamydospore C1C of *Sphaerotheca cruenta* on potato-dextrose agar. The cultures are numbered, according to the position of the sporidia, from the tip of the promycelium to the chlamydospore. The sex is indicated by a plus (+) or (−) sign, according to the pairing reactions shown in Figure 3



Sorghum leaves from plants inoculated with single or monosporidial paired lines of *Sphacelotheca sorghi* (A–C) and *S. cruenta* (D, E) as follows: A, Inoculated with monosporidial line S1A1 (no mottling); B, inoculated with monospordial line S1A2 (no mottling); C, inoculated with sexually compatible lines S1A1 and S1A2 (note mottling); D, inoculated with sexually compatible lines C1A1 and C1B1 (note mottling); E, inoculated with sexually incompatible lines C1A1 and C1B4 (no mottling)

line of C1A paired with monosporidial lines of two additional physiologic forms of *S. sorghi*, namely, S2 and S3.

Apparently the sexual compatibility between interspecific lines also can be determined soon after the plants are inoculated. Certain combinations caused flecking of the plant leaves, as did the sexually compatible pairs of intraspecific lines, and it is significant that only those plants inoculated with the combination that caused flecking of the leaves produced smutty heads. As stated previously, leaf galls occasionally formed on plants inoculated with *Sphacelotheca sorghi*, but in these tests no galls developed as a result of inoculations with interspecific crosses.

Holton (*13*) found that hybrid chlamydospores produced as a result of a cross between *Ustilago levis* and *U. avenae* germinated normally, but that only an occasional primary sporidium developed when iso-

		S1A				C1A			
		1	2	3	4	1	2	3	4
S1A	1	−	+	−	+	+	−	−	+
	2	+	−	+	−	−	+	+	−
	3	−	+	−	+	+	−	−	+
	4	+	−	+	−	−	+	+	−
C1A	1	+	−	+	−	−	+	+	−
	2	−	+	−	+	+	−	−	+
	3	−	+	−	+	+	−	−	+
	4	+	−	+	−	−	+	+	−

FIGURE 5.—Results of inoculating sorghum plants with four monosporidial lines of *Sphacelotheca sorghi* from chlamydospore S1A paired with the four monosporidial lines of *S. cruenta* from chlamydospore C1A

lated. In the present investigations with *Sphacelotheca sorghi* and *S. cruenta* germination tests were made of a large number of primary sporidia isolated from germinating interspecific hybrid chlamydospores. The percentage of germination was found to be just as high as that of sporidia isolated from chlamydospores of inbred lines of either *S. sorghi* or *S. cruenta*. Furthermore, the hybrid chlamydospores were found to be pathogenic, since from 11 to 40 per cent of the Reed kafir plants inoculated with these hybrids developed smutty heads.

Sphacelotheca sorghi and *S. cruenta* have been distinguished macroscopically by the type of membrane surrounding the sori. This structure in the case of *S. sorghi* is relatively thick, grayish brown in color, and seldom ruptured except from mechanical injury. The outer membrane inclosing the sori of *S. cruenta* is thinner and more fragile and ruptures soon after the panicle has emerged, thereby exposing the mass of black chlamydospores. Microscopically, the two species have

been differentiated according to the type of sterile cells produced. Elongated hyaline cells of small diameter, which adhere in chains when the tissue is macerated, are supposed to be typical of *S. sorghi*. The sterile cells reported to be typical of *S. cruenta* are hyaline and spherical, having a diameter about twice that of the chlamydospores, and adhere rather loosely in clumps. In the present investigations, in intraspecific crosses the sori in the panicles were macroscopically characteristic of those of the respective species, whereas in the interspecific crosses sori of the type characteristic of *S. cruenta* were always dominant in the F_1 and the F_2 generations. Microscopic examination of sterile cells revealed the fact that both types of cells were present not only in the interspecific hybrids but also in the intraspecific crosses and in a number of macroscopically distinct specimens of both species. In some sori the sterile cells, which are supposed to be characteristic of the species, predominated; in others the distribution was more even, making it difficult to determine microscopically to which species the smut belonged. Tisdale, Melchers, and Clemmer (*17*) likewise found both types of sterile cells in mature sori of certain collections of the milo and hegari strains of *S. sorghi* and in a number of authentic specimens of both *S. sorghi* and *S. cruenta*. These results indicate that the method of differentiating the two species on the basis of the type of sterile cells produced is not entirely satisfactory.

CONCLUSIONS

From the data presented it is evident that both *Sphacelotheca sorghi* and *S. cruenta* are heterothallic. Monosporidial lines isolated from chlamydospores of the common, milo, and feterita strains of *S. sorghi* and from a collection of *S. cruenta* failed to cause infection of Reed kafir sorghum. When inoculated with sexually compatible lines this variety became infected, as evidenced by the production of chlamydospores in the host.

By mating a monosporidial line of a pathogenically distinct physiologic form of *Sphacelotheca sorghi* with a line of opposite sex of another physiologic form or of another species (*S. cruenta*) hybrid chlamydospores were formed in the host. The fact that these intraspecific and interspecific hybrid chlamydospores have been produced under controlled conditions strengthens the assumption that hybridization accounts, at least in part, for the occurrence of pathogenically distinct strains of the kernel smuts of sorghum. Theoretically these hybrids should contain factors for pathogenicity from both parents, and a recombination of these factors should develop new strains that may differ in virulence from either of the parents. Preliminary tests of the pathogenicity of these hybrids have been made, and the results indicate that the hybrids do differ from either of their parents in ability to infect certain varieties of sorghum.

Ficke and Johnston (*8*) reported that the common, milo, and feterita strains of *Sphacelotheca sorghi* may be distinguished by cultural characteristics on various kinds of nutrient media. In the present investigation, however, it was found that the cultural characteristics of four primary monosporidial lines isolated from a single promycelium of the common strain were distinctly different. In fact, from three chlamydospores isolated from a single smut sorus, 10 of the 12 primary sporidia isolated were distinctly different in type of growth on artificial

media. These results indicate that the common strain of *S. sorghi* can not be distinguished from other pathogenically distinct strains by any one particular type of growth on artificial media.

SUMMARY

Sphacelotheca sorghi (Lk.) Clint. and *S. cruenta* (Kühn) Potter are heterothallic. Monosporidial lines of both species failed to produce chlamydospores in the host plants, but when the plants were inoculated with two monosporidial lines of opposite sex smutted heads were produced.

Monosporidial lines from both *Sphacelotheca sorghi* and *S. cruenta* were found to belong to two sex groups. In intraspecific and interspecific crosses these sex groups were found to be constant and there was no evidence of complete intersterility or interfertility.

Factors determining sex of monosporidial lines of both species segregated on a 2:2 and 1:3 basis. The factors determining sex segregated independently of those determining type of growth of monosporidial lines on artificial media.

Some evidence was obtained indicating that the sexual compatibility of paired lines may be detected soon after the sorghum plants have been inoculated. On leaves of plants inoculated with sexually compatible paired lines distinct chlorotic areas developed four to six days after inoculation, whereas plants inoculated with monosporidial or sexually incompatible lines remained normal.

Sphacelotheca sorghi and *S. cruenta* are interfertile. Intraspecific crosses produced sori in the panicles that were macroscopically characteristic of the species, while interspecific crosses produced sori macroscopically characteristic of *S. cruenta*.

Sterile cells of both the elongated and the spherical type were found to be present in sori resulting from both intraspecific and interspecific crosses.

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PHYSIOLOGIC SPECIALIZATION IN PUCCINIA GRAMINIS SECALIS[1]

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INTRODUCTION

The literature dealing with the physiologic specialization of *Puccinia graminis secalis* Eriks. and Henn. and *P. dispersa secalis* Eriks. and Henn. is exceedingly meager. Levine and Stakman (*4*)[4] reported in 1923 that *P. graminis secalis* comprised at least two, and probably three, distinct physiologic forms, identified by their parasitic effect on three commercial varieties of rye—Rosen, Swedish, and Prolific. Mains (*6*) in 1926 referred to two physiologic forms of *P. dispersa secalis*, which he distinguished by their parasitic effect on an inbred line of Abruzzes (Abruzzi) rye. This selfed line of rye was uniformly highly resistant to his physiologic form 1 and very susceptible to form 2. For obvious reasons the use of standard commercial varieties of cereals as differential hosts in the study of physiologic specialization of the grain rusts is highly desirable. But as rye naturally is cross-pollinated, the study of specialization in *P. graminis secalis* is more difficult than that of other varieties (races) of *P. graminis* for which pure lines of differential hosts may be obtained with comparative ease.

The particular object of the present investigation was to ascertain the number, geographic distribution, and parasitic nature of the physiologic forms of *Puccinia graminis* on rye. An attempt was made to obtain rust collections from as many localities as possible, to identify them, and to determine their parasitic behavior under varying conditions. Particular attention was paid to the rye-growing sections of the United States, the survey being especially concentrated in southeastern Minnesota.

MATERIALS AND METHODS

The first serious endeavor to study intensively physiologic specialization in stem rust of rye was started in 1918. It was not until 1921, however, that the first three differential hosts, mentioned in the introduction, were found. Later, two additional differential varieties, Dakold and Colorless, were added. These five differentials were selected from a dozen or more commercial varieties and inbred lines of rye that had been inoculated repeatedly. In addition to the five differential varieties, each of which reacted differently to different physiologic forms, should be mentioned Giant Winter, obtained from J. F. Brandon, superintendent of the United States Dry-Land Field

[1] Received for publication Dec. 10, 1931; issued September, 1932. Cooperative investigation between the Division of Cereal Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture, and the Agricultural Experiment Station of the University of Minnesota.

[2] Transferred to the Division of Barberry Eradication July 1, 1930.

[3] The writers are indebted to E. C. Stakman, head of the Section of Plant Pathology of the Minnesota Agricultural Experiment Station, and pathologist in the U. S. Department of Agriculture, for suggestions during the course of the investigation. Thanks are due to the various colleagues who provided the writers with much of the inoculation material. The writers are especially appreciative of the cooperation of Albert C. Arny, Division of Agronomy and Plant Genetics, Minnesota Agricultural Experiment Station, for supplying the seed used in this study except as otherwise specified in the text. The history and description of the differential varieties were supplied by H. K. Wilson, assistant professor of agronomy, University of Minnesota, and Manley Champlin, senior professor of field husbandry, University of Saskatchewan.

[4] Reference is made by number (italic) to Literature Cited, p. 314.

Station at Akron, Colo. Giant Winter has proved extremely susceptible to all forms of *Puccinia graminis secalis* so far discovered and consequently can not be properly considered a differential host for these forms.

DESCRIPTION OF DIFFERENTIAL HOSTS

The five commercial varieties of rye chosen as differential hosts are all members of *Secale cereale* L. A brief description of each and of Giant Winter follows:

ROSEN, MINN. ACCESSION No. 82.—Rosen rye was developed at the Michigan Agricultural Experiment Station by selection from Petkus rye obtained from Russia through J. A. Rosen, agricultural expert of the Agrojoint Colonization Committee, in 1909. It was first distributed in 1912. It matures late, has large spikes filled with large dark kernels, is not so winter-hardy as Swedish No. 2, and is well adapted for growing in Michigan, Wisconsin, and southern Minnesota.

SWEDISH, MINN. No. 2.—A very hardy, good-yielding variety, which was produced at the Minnesota Agricultural Experiment Station by selection from a sample of Swedish rye obtained in 1895 from John Brogard, Henning, Minn. Seed was selected from the hardy plants that survived the winter. The seed was first distributed to farmers in 1907, and this is now the leading variety in Minnesota and the eastern part of South Dakota. The plants have small heads with small dark kernels, borne on rather tall, slender culms.

PROLIFIC, MINN. ACCESSION No. 89.—Prolific is a spring rye, the origin of which was a mass selection made at the University of Saskatchewan from an unknown sample of seed received from Germany. It was registered in 1921 under the name "Prolific, Sask. 302," and a quantity of hand-picked seed has been distributed each season since that time. According to Champlin (*2*), it matures very late but is a high yielder and is now grown extensively in the Province of Saskatchewan.

DAKOLD, MINN. ACCESSION No. 93.—Dakold rye was originated by the North Dakota Agricultural Experiment Station in 1902, when a few plants were found in a field where winter wheat had been sown but had been winterkilled. It was first distributed as "N. Dak. No. 959," but was later named Dakold. It resembles Swedish No. 2 in appearance and is very hardy under northwestern conditions. It is widely grown in North Dakota and has produced the highest average yield in a 4-year test at Saskatchewan.

COLORLESS, MINN. No. 104.—Colorless rye was produced at the Minnesota Agricultural Experiment Station by continued, pedigreed selection for the pale-color character from Swedish No. 2, resembling the latter in every other respect.

GIANT WINTER.—Giant Winter rye was introduced into the United States from France by the United States Department of Agriculture in 1901. The variety resembles Swedish No. 2 but appears to be of minor economic importance. It is grown to a certain extent in southeastern Wyoming and northeastern Colorado.

INOCULATION TECHNIC AND CULTURE METHODS

In all, 147 cultures of rye stem rust were studied. Specimens were obtained from 12 States in the United States, extending from Maine to Colorado and from Kansas to Wisconsin; from Ontario, Canada; and from France, Scotland, and Sweden. The hosts on which the rust was orginally found comprised cultivated and volunteer rye, *Secale cereale;* the common barberry, *Berberis vulgaris* L.; and the following wild grasses: *Agropyron repens* (L.) Beauv., *A. smithii* Rydb., *A. tenerum* Vas., *Elymus* sp., *Hordeum jubatum* L., and *H. pusillum* Nutt. By means of inoculations, made for the past decade at University Farm, St. Paul, Minn., 104 of the cultures were separated into distinct physiologic forms. The inoculation technic was essentially that described by Stakman and Piemeisel (*10*). The identity of the forms was determined by a modification of the method used by Stakman and Levine (*8*) in their study of physiologic forms of *Puccinia graminis tritici* Eriks. and Henn. The various types of infection produced by *P. graminis secalis* on rye seedlings were used as a guide in the present study. (Fig. 1.) The host-reaction classes were

adapted with some modification from Levine (*3*). The modifications were necessary because of the heterozygous nature of the differential varieties of rye. This heterozygosity also necessitated, in the majority

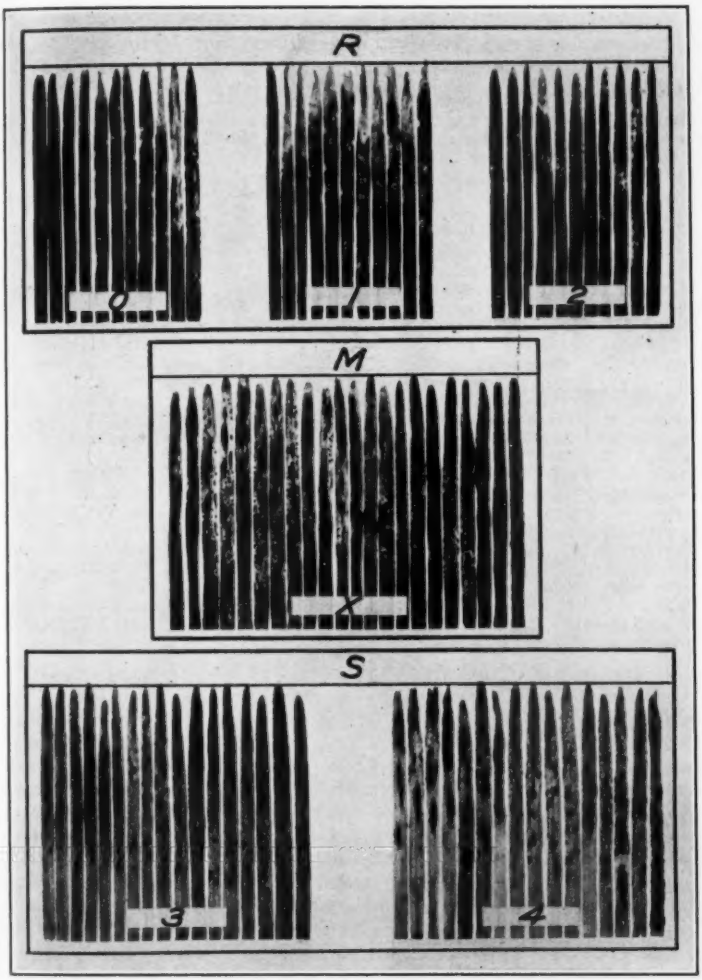

FIGURE 1.—Reaction classes of differential varieties of rye as indicated by infection types produced by *Puccinia graminis secalis:* Class R, resistant, embracing type 0 (no infection whatever or pronounced necrotic flecks), type 1 (minute uredia surrounded by solid necrotic areas), and type 2 (small pustules in green islands surrounded by necrotic halos); class M, mesothetic, consisting of type X (infection heterogeneous and ill defined); class S, susceptible, composed of type 3 (medium-size uredia with slight chlorosis but no necrosis), and type 4 (large confluent uredia, resulting in very heavy infection)

of cases, the testing of rather large numbers of individuals of each differential variety. On an average, more than 200 plants of each variety were infected before the identity of a rust culture was established, although there were some unavoidable exceptions.

CLASSIFICATION OF HOST REACTIONS

Three host-reaction classes—resistant, mesothetic, and susceptible—are recognized in the identification of physiologic forms of *Puccinia graminis secalis*. This classification is based on the different types of infection produced by the rust on seedlings and on the relative number of individuals in a given variety reacting in a definite manner. The following is a schematic description of the reaction classes and infection types:

CLASS R.—A variety is considered resistant when less than 25 per cent of the infected plants show normal development of rust, or conversely, when the rust-infection types produced on 75 per cent or more of the infected plants are, individually or severally, 0, 1, and 2, with their plus and minus fluctuations:

Type 0.—Plants virtually immune; no uredia are developed, but sharply defined necrotic flecks or necrotic lesions usually are present.

Type 1.—Plants extremely resistant; infection very light; uredia minute and scattered, surrounded by clearly defined, continuous necrotic areas.

Type 2.—Plants moderately resistant; infection light; uredia isolated and small to medium in size; hypersensitive areas in the form of necrotic halos or circles; pustules usually in green, though slightly chlorotic, islands.

CLASS M.—The host reaction is considered mesothetic when more than 25 and less than 75 per cent of the infected plants have rust pustules of types 3 and 4, or when the infection on all or most of the plants is heterogeneous as indicated by infection type X with its accompanying plus and minus fluctuations:

Type X.—Infection heterogeneous; uredia very variable, apparently including all types and degrees of infection on the same blade; no mechanical separation seems possible, since on reinoculation spores from small uredia may produce large ones, and vice versa.

CLASS S.—When 75 per cent or more of the infected plants of a given variety are moderately to heavily rusted, i. e., when the infection on them is of either type 3 or 4, or both, including their plus and minus variations, the variety is designated susceptible:

Type 3.—Plants moderately susceptible; infection medium; uredia mid sized with slight tendency to coalesce; true hypersensitiveness absent but light chlorotic areas usually present, especially under unfavorable cultural conditions.

Type 4.—Plants completely susceptible; infection normal and heavy; uredia large and generally confluent; hypersensitiveness normally absent, but chlorosis may be present when cultural conditions are not favorable.

Plus and minus signs indicate a slightly greater or smaller amount of rust than the nearest figure representing the infection type. Necrotic lesions are designated by a dot (.), necrotic flecks by a semicolon (;), and necrotic islands by a colon (:). The sign of equality denotes double minus.

The determination of the rust reaction of a given variety to *Puccinia graminis secalis* is rather simple when a great many plants are tested. The individual plants are examined first for infection types; then they are grouped according to the reaction classes, and the number in each class is recorded. The relative susceptibility of the variety is determined by the quotient obtained from dividing the number of susceptible plants by the total number of infected plants. Thus, for example, Colorless was considered highly susceptible to the Litchfield (Minn.) culture (form 11), because 461, or 91.47 per cent, of the 505 plants that became infected had rust pustules of types 3 and 4; of the remaining infected plants, 43, or 8.51 per cent, were placed in the resistant class, and 1 was classified as mesothetic. Dakold, on the other hand, was considered resistant to this culture, because only 25, or 8.93 per cent, of the 280 infected plants were really susceptible. Similarly, the Swedish variety was classified as mesothetic to the Litchfield culture because 32.08 per cent of the infected plants, i. e., more than 25 but less than 75 per cent, were heavily infected.

However, when the number of plants was unavoidably small, the susceptibility quotient ceased to be effectively operative. In such cases a numerical equivalent, as presented in Table 1, was assigned to each infected plant according to the degree and type of infection produced on it. From these the relative susceptibility of the variety was determined by calculating the average percentage equivalent. For instance, in the case of Rosen, inoculated with the culture from Haddington, Scotland (form 11), 19 plants became infected; 3 of these were marked type 3, 3 were 4−, and the remaining 13 were recorded as 4+. The total numerical equivalent for the 19 plants, according to Table 1, was $3\times50+3\times82+13\times94=1{,}618$, with an average for the variety of $1{,}618\div19$, or 85.16 per cent.

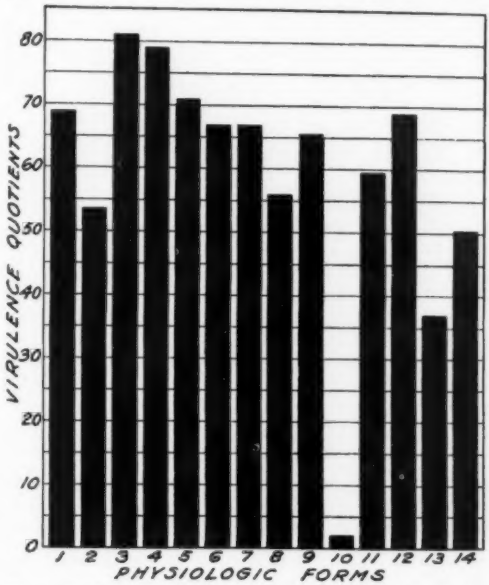

FIGURE 2.—Successive series of the physiologic forms of *Puccinia graminis secalis* indicating their respective virulence quotients

TABLE 1.—*Graduated scale showing numerical equivalents for host reactions, corresponding to symbols representing types and degrees of infection,*[a] *used as a means to determine the relative susceptibility of differential varieties*

Immune to moderately resistant				Mesothetic to extremely susceptible			
Infection index	Numerical equivalent	Infection index	Numerical equivalent	Infection index	Numerical equivalent	Infection index	Numerical equivalent
0	0	x.	8	x=	28	x+	65
0;	½	x;	10	x−	32	x++	70
0:	1	2=	12	x	36	4=	76
1=	2	2−	15	3=	40	4−	82
1−	3	2	18	3−	45	4	88
1	4	2+	21	3	50	4+	94
1+	5	2++	24	3+	55	4++	100
1++	6			3++	60		

[a] For explanation of symbols see p. 300.

EXPERIMENTAL RESULTS

Data regarding the pathogenicity of collections of rust used in these studies are presented in Table 2. In Table 3 are recorded the extreme and mean reactions of the differential varieties and the virulence quotient, of the physiologic forms. The latter are illustrated in Figure 2.

TABLE 2.—*Geographic distribution and parasitic behavior of physiologic forms of Puccinia graminis secalis isolated during the period 1921–1931*

Physiologic form No.	Place of origin	Original host	Stage of rust	Collected by—	Reaction of differential varieties																							
					Rosen					Swedish					Prolific					Dakold					Colorless				
					Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility
					Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible	
									Per cent					*Per cent*					*Per cent*				*Per cent*					*Per cent*
1	St. Paul, Minn.	Secale cereale	Uredial	E. O. Horn, M. N. Levine.	67	55	...	12	17.91	122	12	4	106	86.89	44	2	...	42	95.45	...	...	...	...	...	...	...	...	...	...
2	Madison, Wis.	Agropyron repens.	do	H. B. Humphrey	28	3	3	22	78.57	10	10	...	...	3.40	24	15	...	9	37.50	...	...	...	...	...	...	...	...	...	...
	Massy, France	Secale cereale	do	E. C. Stakman	39	2	...	37	94.87	28	22	...	6	21.43	41	15	9	17	41.46	...	...	...	...	...	...	...	...	...	...
	Caledonia, Ontario.	Agropyron repens.	do	I. L. Conners	15	...	...	15	94.00	27	...	...	27	88.00	16	...	...	16	82.00	...	...	...	...	...	...	...	...	...	...
	Gurnee, Ill.	do	do	J. L. Seal	16	...	...	16	78.44	14	...	...	14	82.21	12	...	...	12	82.00	...	...	...	...	...	...	...	...	...	...
	do	Secale cereale	do	do	15	...	...	15	82.00	14	1	...	13	82.64	18	4	...	14	77.78	...	...	...	...	...	...	...	...	...	...
3	Ithaca, N. Y.	Agropyron repens.	do	R. S. Kirby	26	...	...	26	88.00	15	...	...	15	82.00	18	...	...	18	88.00	...	...	...	...	...	...	...	...	...	...
	Marshall, Wis.	Hordeum jubatum	do	J. L. Seal	10	...	...	10	91.00	12	1	...	11	80.75	18	...	...	18	80.00	...	...	...	...	...	...	...	...	...	...
	Northfield, Minn.	Agropyron repens.	do	J. J. Christensen	23	...	...	23	92.69	11	...	...	11	77.00	12	...	...	12	85.00	...	...	...	...	...	...	...	...	...	...
	Red Wing, Minn	do	do	E. D. Sylvester	21	...	...	21	79.33	15	...	...	15	82.00	13	...	...	13	82.92	...	...	...	...	...	...	...	...	...	...
	Rosemount, Minn.	Berberis vulgaris.	Aecial	F. R. Rasmussen, E. M. Hansing.	377	44	14	319	84.62	471	72	22	377	80.04	306	67	6	233	76.14	458	130	49	279	60.92	386	24	21	341	88.34

	Briançon, France	....do	...do	E. C. Stakman	19			19	84. 21	16			16	88. 00	11	4		7	63. 64															
	Minneapolis, Minn.	Agropyron repens	Uredial	H. C. Gilbert	30	1	3	26	86. 67	11	2		9	81. 82	13	5		8	61. 54															
4	....do	....do	Telial	R. U. Cotter	142	13	1	128	90. 14	210	35	8	167	79. 52	85	17	6	62	72. 94	433	127	46	260	60. 05	184	8		176	95. 65
	Northfield, Minn.	Berberis vulgaris	Aecial	J. J. Christensen	303	15		288	95. 05	213	37		176	82. 63	188	78	1	109	57. 98	127	44		83	65. 34	200	4		196	98. 00
	Commerce, Mich.	Agropyron repens	Uredial	W. F. Reddy, M. N. Levine.	34	5		29	85. 29	17	17			2. 00	18	3		15	83. 33															
5	Presque Isle, Me.	Berberis vulgaris	Aecial	Donald Folsom	20	1		19	82. 30	9	7		2	19. 00	15			15	88. 03															
6	Stockholm, Sweden.	Agropyron repens	Uredial	E. C. Stakman	19	1	2	16	84. 21	31	2		29	93. 55	23	19		4	17. 39															
	Anoka, Minn	Berberis vulgaris	Aecial	L. W. Melander, R. U. Cotter.	155	14	16	125	80. 65	219	75	11	133	60. 73	188	29	32	127	57. 50	370	199	38	133	35. 95	445	62	36	347	77. 98
	Bancroft, S. Dak.	Agropyron repens	Telial	R. O. Bulger	150	11		139	92. 67	108	33		75	69. 44	155	70	3	82	52. 90	163	112	5	46	28. 22	137	6		131	95. 62
	Belleville, Kans.	Agropyron smithii	Uredial	D. G. Fletcher	213	17		196	92. 02	129	47		82	63. 57	161	66	2	93	57. 76	279	179	9	91	32. 62	149	7		142	95. 30
	Blooming Prairie, Minn.	Berberis vulgaris	Aecial	L. W. Melander, J. M. Walter.	190	18	5	167	87. 89	343	100	33	210	61. 22	156	34	11	111	71. 15	478	212	52	214	44. 77	357	29	16	312	87. 39
	Bloomington, Wis.	Secale cereale	Uredial	W. J. Seymour	321	7		314	97. 82	348	112		236	67. 82	287	102		185	64. 46	192	108		84	43. 75	254	13		241	94. 88
	Charlevoix, Mich.	Agropyron repens	Telial	W. F. Reddy	168	12	1	155	92. 26	118	41		77	65. 25	248	107	14	127	57. 21	136	76	5	55	40. 44	329	15		314	95. 44
	Chatham, Mich	....do	...do	B. R. Churchill	170	3	2	165	97. 06	487	152	4	331	67. 96	342	99		243	71. 05	285	164	5	116	40. 70	238	1		237	99. 58
	Chatfield, Minn.	....do	...do	L. W. Melander	290	14	29	247	85. 17	549	216	70	263	47. 91	238	74	25	139	58. 40	661	420	68	173	25. 17	585	18	50	517	88. 38
	Cherrygrove, Minn.	Berberis vulgaris	Aecial	V. F. Peterson, B. C. Dettman.	253	13		240	94. 86	268	106	2	160	59. 70	416	172		244	58. 65	323	224	2	97	30. 03	228	16		212	92. 98
	Decorah, Iowa	Agropyron repens	Telial	P. W. Rohrbaugh, L. W. Melander.	192	12	4	176	91. 67	385	85	69	231	60. 00	168	56	12	100	59. 52	517	270	97	150	29. 01	261	21	21	219	83. 91
	Faribault, Minn.	Berberis vulgaris	Aecial	L. W. Melander, J. J. Christensen.	479	57	32	390	81. 42	434	81	42	311	71. 65	302	68	38	196	64. 90	529	177	87	265	50. 09	591	71	36	484	81. 90
	Fond du Lac, Wis.	....do	...do	E. C. Barsch	601	30	80	491	81. 70	544	127	100	317	58. 27	267	64	84	119	44. 57	840	392	163	285	33. 93	580	26	106	448	77. 24
7	Fort Collins, Colo.	Agropyron tenerum.	Telial	E. A. Lungren	61	8		53	86. 89	43	21		25	54. 35	130	50	1	79	60. 77	94	60	3	31	32. 98	178	22		156	87. 64
	Ganges, Mich	Agropyron repens	Uredial	E. A. Weimer	381	2		379	99. 48	374	114	2	258	68. 98	255	95		160	62. 75	432	213	23	196	45. 37	346	41		305	88. 15
	Gilman City, Mo.	Hordeum jubatum	...do	D. G. Fletcher	256	29		227	88. 67	220	83	4	133	60. 45	382	146	3	233	60. 99	164	93		71	43. 29	479	44		435	90. 81
	Lake City, Minn.	Agropyron repens	Telial	L. W. Melander, R. U. Cotter.	271	11		260	95. 94	103	27	6	70	67. 96	140	51	4	85	60. 71	106	52	9	45	42. 45	287	2		285	99. 30
	Lebanon, Kans.	Hordeum pusillum.	Uredial	D. G. Fletcher	197	4		193	97. 97	356	97	14	245	68. 82	286	112	4	170	59. 44	247	137	2	108	43. 72	119	8		111	93. 28
	Lesueur Center, Minn.	Agropyron repens	...do	H. B. Humphrey, L. W. Melander.	127	22		105	82. 68	501	105	20	376	68. 74	625	217	10	398	63. 68	429	256	2	171	39. 86	262	13		249	95. 04
	Lewiston, Minn.	Secale cereale	...do	L. H. Person	154	3		151	98. 05	201	68	2	133	66. 17	150	64		86	57. 33	115	57	11	47	40. 87	113	7		106	93. 81
	Lydia, Minn	Berberis vulgaris	Aecial	F. R. Rasmussen, W. C. Hanson.	502	81	15	406	80. 88	300	94	4	202	67. 33	202	95	3	104	51. 49	317	137	10	170	53. 63	284	39	1	244	85. 92
	Plainview, Minn.	Agropyron repens	Uredial	V. F. Peterson, B. C. Dettman.	188	9		179	95. 21	297	105	15	177	59. 60	220	100	6	114	51. 82	184	85	12	87	47. 28	124	12		112	90. 32
	Red Wing, Minn.	....do	Telial	L. W. Melander, R. U. Cotter.	158	8		150	94. 94	184	77	7	100	54. 35	157	54	4	99	63. 06	165	108		57	34. 55	273	26	4	243	89. 01
	....do	Berberis vulgaris	Aecial	M. N. Kelso, H. G. Ukkelberg.	61	4		57	93. 44	25	6		19	51. 24	61	18		43	70. 49	88	58		30	34. 09	133	8		125	93. 98

TABLE 2.—*Geographic distribution and parasitic behavior of physiologic forms of Puccinia graminis secalis isolated during the period 1921–1931*—Continued

Physiologic form No.	Place of origin	Original host	Stage of rust	Collected by—	Reaction of differential varieties																							
					Rosen					Swedish					Prolific					Dakold					Colorless				
					Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility
					Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible	
									Per cent					*Per cent*					*Per cent*									
7	Rochester, Minn.	Agropyron repens	Telial	L. W. Melander	143	15	1	127	88.81	146	75	2	69	47.26	207	76	33	9	847.34	645	298	140	207	32.09	512	16	50	446	87.11
	Salina, Kans.	Agropyron smithii	Uredial	D. G. Fletcher	52	4		48	92.31	91	51		40	43.96	165	63		10	261.82	45	32		13	28.89	213	11	1	201	94.37
	Saratoga, Minn.	Agropyron repens	do	L. W. Melander	110	8	1	101	91.82	327	157		170	51.99	338	96	1	24	171.30	156	104	1	51	32.69	211	10		201	95.26
	Sauk Center, Minn.	do	do	C. L. Lefebvre	196	45		151	77.04	239	65	12	162	67.78	304	125	4	17	557.57	348	165	18	165	42.99	468	52		416	88.89
	Belleville, Kans.	Elymus sp	do	G. L. Peltier	164	74		90	54.88	133	91	1	41	30.83	341	131	4	20	660.41	233	196	4	33	14.16	357	77		280	78.43
	do	Hordeum pusillum	do	F. J. Scrivner	233	60		173	74.25	352	201		151	42.90	287	120		16	758.19	150	134		16	10.67	153	17		136	88.89
8	Goodhue, Minn.	Secale cereale	do	L. W. Melander	244	29	56	159	65.16	147	69	2	76	51.70	436	136	2	29	868.35	115	84	9	22	19.13	328	52	29	247	75.31
	Invergrove, Minn.	Berberis vulgaris	Aecial	S. P. Swenson, C. P. Shumway	310	58	24	228	73.55	159	70	21	68	42.77	156	55	8	9	258.97	253	207	15	31	12.25	251	30	19	202	80.48
	Presque Isle, Me.	Agropyron repens	Uredial	Donald Folsom	94	32	4	58	61.70	88	59	1	28	31.82	103	34	10	5	957.28										
	Cherrygrove, Minn.	Berberis vulgaris	Aecial	L. F. Wasson, W. D. Swenson.	481	50	25	406	84.41	534	143	61	330	61.80	516	44	34	43	884.86	641	452	53	136	21.22	473	34	24	415	87.74
	Decorah, Iowa	Agropyron repens	Telial	P. W. Rohrbaugh, L. W. Melander.	235	21		214	91.06	208	109	5	94	45.19	247	37	5	20	583.00	458	365	6	87	19.00	209	38	13	158	75.60
	Kenyon, Minn.	Berberis vulgaris	Aecial	L. F. Wasson, W. D. Swenson.	357	40	5	312	87.12	241	120	23	98	40.66	141	21	4	11	682.27	315	239	13	63	20.00	333	26	18	289	86.79
9	Marengo, Ill.	Agropyron repens	Telial	I. L. Brakansiek, L. J. Ham.	102	9		93	91.08	186	89	2	95	51.08	175	38		13	778.29	107	88		19	17.76	326	43	8	275	84.36
	Ponca, Nebr.	Secale cereale	Uredial	A. F. Thiel	144	32		112	77.78	174	94		80	48.41	148	28	4	11	678.38	140	115		25	17.86	203	26		177	87.19
	Union Lake, Minn.	Berberis vulgaris	Aecial	L. W. Melander, J. J. Christensen.	318	23	8	230	91.19	280	88	29	163	58.21	187	25	9	15	381.82	312	221	21	70	22.44	503	19	11	473	94.04
	Wanamingo, Minn.	Agropyron repens	Telial	L. W. Melander	185	21		164	88.65	200	105		95	47.50	369	66	20	28	376.89	152	135		17	11.18	505	70	9	426	84.36

10	Akron, Colo	Secale cereale	Uredial	M. N. Levine	26	26			1.85	18	18			2.22	16	16			2.50																	
	Belleville, Kans	Berberis vulgaris	Aecial	D. G. Fletcher	175	25		150	85.71	377	208	1	168	44.56	136	79		57	41.91	332	264		68	20.48	146	10		136	93.15
	do	do	do	G. H. Starr	399	51	16	332	83.21	220	125	4	91	41.36	288	103	1	184	63.89	292	236	13	43	14.73	203	20	2	181	89.16
	do	Hordeum jubatum	Uredial	G. L. Peltier	162	20		142	87.65	204	100		104	50.98	276	107	11	158	57.25	278	211	16	51	18.35	379	52	5	322	84.96
	Chatfield, Minn	Agropyron repens	Telial	L. W. Melander	651	82	2	567	87.10	1,038	369	94	575	55.39	742	157	96	489	65.90	1,091	773	74	244	22.36	1,514	118	83	1,313	86.72
	Decorah, Iowa	do	do	P.W. Rohrbaugh, L.W. Melander.	250	9		241	96.40	237	91	5	141	59.49	165	56	3	106	64.24	347	272	18	57	16.43	235	13	2	220	93.62
	East Lansing, Mich.	Elymus sp.	do	W. F. Reddy	144	4		140	97.22	126	58	1	67	53.14	166	73		93	56.02	171	127	2	42	19.52	236	22		214	90.68
	Faribault, Minn	Berberis vulgaris	Aecial	L. W. Melander, J.J. Christensen.	574	94	25	455	79.27	267	145	15	107	40.07	223	85	16	122	54.71	465	405	15	45	9.68	466	64	28	374	80.26
	Haddington, Scotland.	Agropyron repens	Uredial	E. C. Stakman	19			19	85.16	11		6	5	43.82	24		13	11	42.42																	
	Ishpeming, Mich	Berberis vulgaris	Aecial	H. S. Hunter	385	25	24	336	87.27	238	110	14	114	47.90	90	32	5	53	58.89	308	260	19	29	9.42	248	27	8	213	85.88
	Litchfield, Minn	Hordeum jubatum	Telial	L. W. Melander, R. U. Cotter.	143	22	2	119	84.40	427	260	30	137	32.08	288	166	7	115	39.93	280	255		25	8.93	505	43	1	461	91.47
	Loyalton, S. Dak	Agropyron repens	Uredial	G.W. Eade, H. C. Batson, et al.	413	38	28	347	84.02	384	187	32	165	42.97	293	72	30	191	65.19	556	467	21	68	5.56	378	37	36	305	80.69
	do	Berberis vulgaris	Aecial	G. W. Eade	361	43	11	307	85.04	287	108	9	170	59.23	303	83	24	196	64.69	303	239	9	55	18.15	380	34	28	318	83.68
	Mankato, Minn	do	do	F. R. Rasmussen	336	33	26	277	82.44	257	110	14	133	51.75	207	49	17	141	68.12	323	247	21	55	17.03	370	36	7	327	88.38
	Marquette, Mich	do	do	H. S. Hunter	328	33	11	284	86.58	225	68	56	101	44.89	118	34	6	78	66.11	270	168	59	43	15.85	222	21	19	182	81.98
	Minneapolis, Minn.	Agropyron repens	Telial	R. U. Cotter	223	30		193	86.55	266	156		110	41.35	173	80	3	90	52.02	220	182	1	37	16.82	175	24		151	86.29
	do	do	do	do	239	20	1	218	91.21	189	102	2	85	44.97	194	72		122	62.89	222	198	1	23	10.36	291	24	1	266	91.41
11	do	do	do	D. G. Fletcher	113	1		112	99.11	199	90	8	101	50.75	90	50	1	39	43.33	256	195	7	54	16.70	102	8		94	92.15
	Minnetrista, Minn.	do	do	L. W. Melander, F. R. Rasmussen.	102	4		98	96.08	130	72	6	52	40.00	140	45	10	85	60.71	253	194		59	23.32	275	36	6	233	84.73
	New Brighton, Minn.	do	do	R. U. Cotter	50	1		49	98.00	186	86	21	79	42.47	86	27	3	56	65.12	217	158	18	41	18.89	122	7	4	111	90.98
	Orono, Minn	do	do	L. W. Melander	204	17		187	91.67	196	117	3	76	38.78	138	66		72	52.17	164	141	3	20	12.20	206	20	4	182	88.35
	Petoskey, Mich	Secale cereale	Uredial	R. E. Warner	99	18		81	81.82	78	47	2	29	37.18	72	44	1	27	37.50	52	49		3	5.77	116	27		89	76.72
	Redding, Iowa	Hordeum jubatum	do	D. G. Fletcher	156	15		141	90.38	135	59	19	57	42.23	181	77		104	57.46	137	113		24	17.52	379	82		297	79.41
	Rochester, Minn	Agropyron repens	Telial	L. W. Melander	245	9	8	228	93.13	260	123	39	98	37.69	202	55	45	102	50.50	272	199	45	28	10.29	483	17	75	391	80.95
	do	do	do	L. W. Melander, R. U. Cotter.	118	20		98	83.05	561	268	17	276	49.20	330	128	9	193	58.48	117	103		14	11.97	383	64		319	83.29
	Rochester, N. Y	do	Uredial	R. S. Kirby	155	27		128	82.58	101	57	7	37	36.63	47	27		20	42.55	32	27		5	15.63	28	2		26	92.86
	Rosetown, Mian	do	Telial	R. U. Cotter	173	7		166	95.95	396	186	23	187	47.22	254	67	5	182	71.65	511	377	18	116	22.70	468	41	10	417	89.10
	St. Paul, Minn	do	do	do	153	17		136	88.89	201	74	10	117	58.21	125	52		73	58.40	344	251	15	78	22.67	572	55	5	512	89.51
	do	Secale cereale	Uredial	H. E. Brewbaker	61	7		54	88.52	69	23	7	39	56.52	41	11	2	28	68.29																	
	Salina, Kans	Berberis vulgaris	Aecial	W. A. Walker	135	16		119	88.02	731	291	198	242	33.11	249	108	10	131	52.61	187	158	6	23	12.30	354	32		322	90.96
	Traverse City, Mich.	Secale cereale	Uredial	R. G. Smith	239	9		230	96.23	309	137		172	55.66	192	95		97	50.52	133	98		35	22.97	215	14		201	93.49
	Wells, Minn	Agropyron smithii	do	C. L. Lefebvre, V. F. Peterson.	168	24	7	137	81.55	201	102	16	83	41.29	223	80	14	129	57.85	143	123		20	13.99	497	40	2	455	91.55
	Woodstock, Ill	Hordeum jubatum	do	L. R. Davis	209	32		177	84.69	307	101	16	190	61.89	210	71	9	130	61.90	387	258	46	83	21.19	599	87	5	507	84.64
	Zumbra Heights, Minn.	Agropyron repens	Telial	L. W. Melander	106	8		98	92.45	95	37	6	52	54.74	140	45	10	85	60.71	253	194		59	23.32	275	36	6	233	84.73

TABLE 2.—*Geographic distribution and parasitic behavior of physiologic forms of Puccinia graminis secalis isolated during the period 1921–1931*—Continued

Physiologic form No.	Place of origin	Original host	Stage of rust	Collected by—	Reaction of differential varieties																							
					Rosen					Swedish					Prolific					Dakold					Colorless			
					Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility	Number of plants				Degree of relative susceptibility
					Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible		Total infected	Resistant	Mesothetic	Susceptible	
									Per cent					*Per cent*					*Per cent*					*Per cent*				
	Haycreek, Minn.	Berberis vulgaris	Aecial	J. J. Christensen, L. W. Melander.	360	39	22	299	83.06	344	68	71	205	59.59	290	43	24	223	76.90	613	294	112	207	33.35	327	27	23	277	84.71
	Invergrove, Minn.	do	do	P. L. Holmberg, C. Marti.	273	18	2	253	92.67	292	68	32	192	65.75	113	10	4	99	87.61	224	140	15	69	30.80	286	10	...	276	96.50
	Minneapolis, Minn.	Agropyron repens	Telial	D. G. Fletcher	44	4	...	40	90.91	20	7	...	13	65.00	9	...	...	9	82.00	46	15	...	31	67.39	50	1	...	49	98.00
12	do	Hordeum jubatum	Uredial	H. C. Gilbert	17	...	...	17	88.00	11	...	11	...	65.00	16	...	...	16	82.00	...	...	...	...	...	...	...	...	...	...
	Northfield, Minn.	Agropyron repens	do	F. R. Rasmussen	477	50	20	407	85.32	516	125	37	354	68.60	375	56	16	303	80.80	727	367	68	292	40.17	487	34	22	431	88.50
	do	Hordeum jubatum	do	do	407	18	41	348	85.50	507	73	75	359	70.81	349	30	23	336	86.38	605	173	143	289	47.77	516	38	41	437	84.46
	Ord, Nebr.	Berberis vulgaris	Aecial	G. A. Feather	255	22	30	203	79.61	289	67	67	155	53.63	117	9	19	89	76.07	261	135	56	70	26.82	309	19	38	252	81.55
	Potosi, Wis.	Secale cereale	Uredial	L. G. Strom	468	27	36	405	86.54	401	65	90	246	61.35	316	34	33	249	78.80	454	191	122	141	31.06	433	17	21	395	91.22
	Redfield, S. Dak.	do	do	M. N. Levine	119	10	...	109	91.60	63	25	...	38	60.32	31	5	...	26	83.87	43	26	...	17	39.53	221	28	...	193	87.33
	Westfield, Minn.	Berberis vulgaris	Aecial	J. M. Walter, L. W. Melander.	211	25	3	183	86.73	212	74	13	125	58.96	274	39	18	217	79.20	308	196	23	89	28.90	263	24	10	229	87.07
13	Morris, Minn.	Secale cereale	Uredial	M. N. Levine	280	153	24	103	36.79	447	331	23	93	20.80	365	174	18	173	47.40	342	317	9	16	4.68	565	164	42	359	63.54
	Brookings, S. Dak.	Berberis vulgaris	Aecial	R. O. Bulger	310	41	2	267	86.13	359	214	1	144	40.11	214	85	...	129	60.28	316	281	3	32	10.13	183	54	...	129	70.49
14	Central Lake, Mich.	Agropyron repens	Telial	W. F. Reddy	48	8	...	40	83.33	100	61	...	39	39.00	137	77	...	60	43.79	72	62	...	10	13.88	167	57	...	110	65.87

TABLE 3.—*Relative susceptibility of differential varieties of Secale cereale and comparative virulence of physiologic forms of Puccinia graminis secalis*

Physiologic form No.	Relative susceptibility of—										Virulence quotient
	Rosen		Swedish		Prolific		Dakold		Colorless		
	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	
1	0.43–24.87	17.91	74.54–94.00	86.89	82.47–98.00	95.45					68.67
2	78.57–94.87	88.06	3.40–21.43	15.79	37.50–41.46	40.00					53.53
3	78.44–94.00	88.47	77.00–88.00	83.42	76.14–88.00	81.36	56.34–71.43	60.92	84.50–91.67	88.34	80.55
4	84.21–95.05	93.32	79.52–88.00	81.78	57.98–72.94	62.63	60.05–65.34	61.25	95.65–98.00	96.88	79.18
5	82.30–85.29	84.29	2.00–19.00	6.86	83.33–88.00	85.67					70.80
6	82.00–94.00	84.21	88.00–96.00	93.55	.87–22.75	17.39					67.12
7	77.04–99.48	89.29	43.96–71.66	62.72	44.57–71.30	60.35	26.17–53.63	38.01	77.24–99.58	88.76	66.90
8	54.88–74.25	67.75	30.83–51.70	41.41	57.28–68.35	62.13	10.67–19.13	13.58	75.31–88.89	79.43	56.24
9	77.78–91.19	87.32	40.66–61.80	52.39	76.89–84.86	81.21	11.18–22.44	19.62	75.60–94.04	86.72	65.55
10	1.00– 2.00	1.85	1.00– 3.00	2.22	1.00– 3.00	2.50					2.13
11	79.27–99.11	87.35	32.08–61.89	46.70	37.50–71.65	58.68	5.56–23.32	17.35	76.72–93.62	86.60	59.53
12	79.61–92.67	86.05	53.63–70.81	63.54	76.07–87.61	81.20	26.82–67.39	36.73	81.55–98.00	87.79	69.18
13	25.58–56.49	36.79	15.37–24.13	20.80	31.25–71.96	47.40	2.53–13.24	4.68	49.75–71.27	63.54	37.22
14	83.33–86.13	85.75	39.00–40.11	39.87	43.79–60.28	53.85	10.13–13.88	10.82	65.87–70.49	68.29	50.37
Weighted average	85.88		54.85		63.96		28.29		86.55		63.57

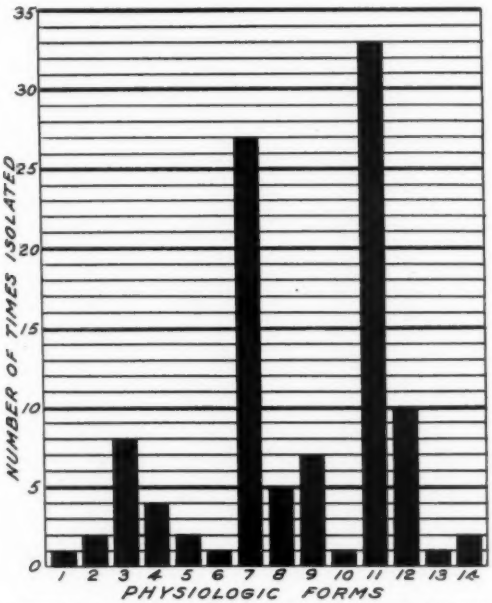

FIGURE 3.—Frequency distribution of physiologic forms of *Puccinia graminis secalis* identified during the period 1921–1931

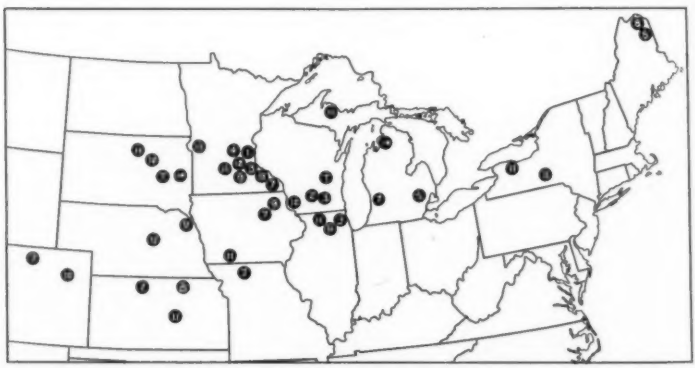

FIGURE 4.—Distribution of physiologic forms (numerals in circles) of *Puccinia graminis secalis* in the United States as represented by individual collections made and identified during the period 1921–1931

Data on the prevalence and distribution of the physiologic forms are presented in Figures 3 and 4 and in the following list:

Places where the different physiologic forms of Puccinia graminis secalis were collected, 1921–1931, and the number of times each was isolated

	Times isolated
Form 1	1
1921—St. Paul, Minn.	
Form 2	2
1922—Madison, Wis.	
Massy, France.	
Form 3	8
1921—Gurnee, Ill.	
1922—Caledonia, Ontario.	
Gurnee, Ill.	
Ithaca, N. Y.	
Marshall, Wis.	
Northfield, Minn.	
Red Wing, Minn.	
1931—Rosemount, Minn.	
Form 4	4
1922—Briançon, France.	
Minneapolis, Minn.	
1927—Northfield, Minn.	
1930—Minneapolis, Minn.	
Form 5	2
1922—Presque Isle, Me.	
1923—Commerce, Mich.	
Form 6	1
1922—Stockholm, Sweden.	
Form 7	27
1925—Gilman City, Mo.	
Red Wing, Minn.	
1926—Bancroft, S. Dak.	
Charlevoix, Mich.	
Chatham, Mich.	
Fort Collins, Colo.	
Lake City, Minn.	
Sauk Center, Minn.	
1927—Belleville, Kans.	
Bloomington, Wis.	
Cherrygrove, Minn.	
Decorah, Iowa.	
Ganges, Mich.	
Lebanon, Kans.	
Lesueur Center, Minn.	
Lewiston, Minn.	
Plainview, Minn.	
Red Wing, Minn.	
Salina, Kans.	
Saratoga, Minn.	
1929—Chatfield, Minn.	
Rochester, Minn.	
1930—Lydia, Minn.	
1931—Anoka, Minn.	
Blooming Prairie, Minn.	
Faribault, Minn.	
Fond du Lac, Wis.	
Form 8	5
1922—Presque Isle, Me.	
1924—Belleville, Kans.	
Goodhue, Minn.	
1927—Belleville, Kans.	
1931—Invergrove, Minn.	
Form 9	7
1924—Wanamingo, Minn.	
1926—Marengo, Ill.	
Ponca, Nebr.	
1927—Decorah, Iowa.	
1931—Cherrygrove, Minn.	
Kenyon, Minn.	
Union Lake, Minn.	
Form 10	1
1923—Akron, Colo.	
Form 11	33
1922—Haddington, Scotland.	
Rochester, N. Y.	
St. Paul, Minn.	
1924—Redding, Iowa.	
1925—Belleville, Kans.	
Litchfield, Minn.	
1926—East Lansing, Mich.	
Minneapolis, Minn.	
Petoskey, Mich.	
Salina, Kans.	
Wells, Minn.	
Woodstock, Ill.	
1927—Belleville, Kans.	
Decorah, Iowa.	
Rochester, Minn.	
Traverse City, Mich.	
1928—New Brighton, Minn.	
Zumbra Heights, Minn.	
1929—Chatfield, Minn.	
Minnetrista, Minn.	
Orono, Minn.	
Rochester, Minn.	
Rosetown, Minn.	
St. Paul, Minn.	
1930—Minneapolis, Minn. (twice)	
1931—Belleville, Kans.	
Faribault, Minn.	
Ishpeming, Mich.	
Loyalton, S. Dak. (twice).	
Mankato, Minn.	
Marquette, Mich.	
Form 12	10
1922—Minneapolis, Minn.	
1925—Redfield, S. Dak.	
1928—Minneapolis, Minn.	
1931—Haycreek, Minn.	
Invergrove, Minn.	
Northfield, Minn. (twice).	
Ord, Nebr.	
Potosi, Wis.	
Westfield, Minn.	
Form 13	1
1926—Morris, Minn.	
Form 14	2
1926—Central Lake, Mich.	
1927—Brookings, S. Dak.	

The number of forms identified in each year was as follows: In 1921, 2; in 1922, 8; in 1923, 2; in 1924, 3; in 1925, 3; in 1926, 5; in 1927, 6; in 1928, 2; in 1929, 2; in 1930, 3; in 1931, 6; total, 14.

It is noteworthy that each of all but two of the collections studied consisted of a single physiologic form. The exceptions were telial collections on *Agropyron repens*, one from Decorah, Iowa, and the other from Chatfield, Minn. *Berberis vulgaris* was inoculated with sporidia of these collections, and the resulting aecia yielded three distinct forms from the Decorah collection (forms 7, 9, and 11) and two forms from the Chatfield collection (forms 7 and 11).

PHYSIOLOGIC SPECIALIZATION

When the type and degree of infection and the relative susceptibility of each differential variety have been determined, the physiologic forms are identified with the aid of a key similar to those used for the identification of physiologic forms of *Puccinia graminis tritici* and *P. graminis avenae* (*8*, *1*). The key is here included. Only the reaction class of each variety is considered. For example, if Rosen is resistant and Swedish susceptible, the key would indicate that the culture producing this reaction is *P. graminis secalis* form 1. On the other hand, if Rosen is susceptible, Swedish and Prolific mesothetic, Dakold resistant, and Colorless susceptible, the rye stem rust causing these reactions would be identified as form 11. The key does not indicate the reaction of the complete set of differentials in every instance. It is therefore necessary to check the tentative identification with the known action of the corresponding forms as indicated in Table 3. If the relative susceptibility of each of the differential hosts approximates that designated for the physiologic form in question, the identification is considered complete; if it does not, then either the form has not yet been described or the culture consists of a mixture of forms, which must be separated before correct identification can be made.

Identification of Physiologic Forms

Analytical key for the identification of physiologic forms of *Puccinia graminis secalis* on the basis of their parasitic behavior on five differential varieties within the species *Secale cereale*.

	Form No.
Rosen resistant:	
Swedish resistant	10
Swedish susceptible	1
Rosen mesothetic:	
Swedish resistant	13
Swedish mesothetic	8
Rosen susceptible:	
Swedish resistant—	
Prolific mesothetic	2
Prolific susceptible	5
Swedish mesothetic—	
Prolific mesothetic—	
Dakold resistant—	
Colorless mesothetic	14
Colorless susceptible	11
Dakold mesothetic	7
Prolific susceptible—	
Dakold resistant	9
Dakold mesothetic	12
Swedish susceptible—	
Prolific resistant	6
Prolific mesothetic	4
Prolific susceptible	3

PATHOGENICITY

It will be seen from Tables 2 and 3 that some of the physiologic forms of *Puccinia graminis secalis* are very virulent, whereas others are extremely weak on the differential varieties of rye. The behavior of the first 3 of the 14 identified forms on Rosen, Swedish, and Prolific has been briefly described by Levine and Stakman (*4*) as follows:

> One form [form 3] is quite virulent, possessing the capability of attacking heavily all of the three varieties; another form [form 2] attacks Rosen very heavily, Prolific only moderately and Swedish very weakly; while still another form [form 1] produces normal infection on Swedish and Prolific, but only weak infection on Rosen.

Judging from its virulence quotient (Table 3 and fig. 2), it would seem that form 3 is the most virulent of all known forms, but only slightly more so than form 4. Next in order of virulence is form 5. It must be remembered, however, that the reaction of Dakold and Colorless to this form is unknown; consequently its exact virulence quotient is not fully established. Form 12, with a virulence quotient of 69.18, follows very closely behind form 5, whose quotient is 70.80 on the basis of the three varieties tested. Each of these quotients is considerably lower than that of form 3, whose quotient is 80.55, or that of form 4, whose quotient is 79.18. Unlike form 5, forms 3, 4, and 12 have been tested on all of the differentials. The weakest in virulence, as far as has been determined, is form 10, whose quotient is only 2.13, the next being form 13 with a quotient of 37.22. However, it must be borne in mind that form 10, like form 5, has been tested on only three differential hosts.

PREVALENCE AND DISTRIBUTION

More than half of all collections identified consisted of only forms 7 and 11. These two forms in the main closely resemble each other in their pathogenicity on the five differentials. The only outstanding difference is furnished by Dakold, a variety mesothetic to Form 7 and resistant to form 11. The difference in virulence quotient of the two forms is slightly more than 7 points. Forms 7 and 11 resemble each other not only in frequency distribution, 27 and 33 isolations respectively (see p. 309, Table 4, and fig. 3), but also in extent of distribution geographically. Both forms were for the most part found in only three States—Kansas, Michigan, and Minnesota (Table 4); form 7 was collected in these States twenty-one times, and form 11, twenty-six times. In addition, form 7 was found also in Colorado, Iowa, Missouri, South Dakota, and Wisconsin; while form 11 was collected also in Illinois, Iowa, New York, South Dakota, and Scotland. Form 7 was isolated twice in 1925, six times in 1926, twelve times in 1927, twice in 1929, once in 1930, and four times in 1931, whereas form 11 appeared three times in 1922, once in 1924, twice in 1925, six times in 1926, four times in 1927, twice in 1928, six times in 1929, twice in 1930, and seven times in 1931.

TABLE 4.—*Prevalence and distribution of physiologic forms of Puccinia graminis secalis identified during the period 1921–1931*

Distribution	Number of times indicated form was found														Total number of—	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Cultures	Forms
United States:																
Colorado							1			1					2	2
Illinois			2						1		1				4	3
Iowa							1		1		2				4	3
Kansas							3	2			4				9	3
Maine					1			1							2	2
Michigan					1		3				5			1	10	4
Minnesota	1		3	3			15	2	4		17	7	1		53	9
Missouri							1					1			1	1
Nebraska									1						2	2
New York			1								1	1			2	2
South Dakota							1				2	1		1	5	4
Wisconsin		1	1				2								5	4
Foreign countries:																
Canada			1												1	1
France		1		1											2	2
Scotland											1				1	1
Sweden						1									1	1
Total	1	2	8	4	2	1	27	5	7	1	33	10	1	2	104	14

The only other forms that were fairly common, besides those just discussed, were forms 3 and 12. Form 3 was isolated only during three years of the investigation, once in 1921, six times in 1922, and once in 1931, but it occurred in New York, also through Ontario to Minnesota, in an almost straight line. Form 12 was found once each in 1922, 1925, and 1928, but was isolated seven times in 1931. As is true of the physiologic forms of stem rusts of wheat and of oats (*3*, *5*, *9*), the same forms of *Puccinia graminis secalis* did not recur with any regularity year after year, nor were they uniformly distributed in any definite area.

All the forms except form 6 have been found in the United States. Form 6 came from Stockholm, Sweden. However, since this study by no means constitutes a complete survey of the status of rye stem rust, it is unlikely that all of the forms that may have been present were collected.

DISCUSSION AND CONCLUSIONS

Notwithstanding the heterozygous nature of commercial varieties of rye, it was possible to establish the existence of at least 14 physiologic forms of *Puccinia graminis secalis* by the use of certain standard varieties of *Secale cereale*. In many cases great numbers of plants must be tested to be certain of the results. However, when the results of inoculation are clear-cut and uniform, smaller numbers may suffice. In the identification of form 7, for example, where three of the five differential varieties are mesothetic or intermediate in reaction, not less than 200 seedlings of each variety were found to be necessary. In the case of form 10, on the other hand, apparently reliable results were obtained with a tenth of the required number of plants tested. The infection types in the last instance were very definite and absolutely uniform. The maximum infection on any one plant was (1−), the minimum (0;). It seemed reasonable to accept these results as conclusive and as a basis for considering the

existence of form 10 as highly probable. That this form is a component part of *P. graminis secalis* is evidenced by the fact that the original culture came from heavily infected Giant Winter rye. At any rate, the majority of the forms described in this paper were identified after a considerable number of individuals of the various differential hosts had been tested. Forms 7 and 11 were found most widely distributed and of most frequent occurrence, representing about 26 and 32 per cent, respectively, of the total number of cultures identified. Form 12 was third, with a 10 per cent ratio in the frequency distribution, and form 3 was fourth, with approximately 8 per cent of the total number identified. The remaining 11 forms gradually tapered down from seven isolations to only a single one.

Seventy-four of the collections were identified directly from the original material, for they were either in the uredial (46 cultures) or aecial (28 cultures) stages. The remaining 30 cultures were of telial origin and had to pass through the aecial stage on barberry (*Berberis vulgaris* L., *B. oblonga* Schn., or *B. notabilis* Schn.) before their behavior on the differentials could be ascertained. It was from two telial collections on quack grass that three different forms (7, 9, and 11), in one case, and two forms (7 and 11), in another case, were isolated on passing through the aecial stage on the common barberry. Inasmuch as it is not known whether the original material in either case consisted of a single physiologic form, it would be hazardous to assume that a genetic change had occurred. There is evidence, however, of hybridization in the aecial stage, resulting from crossing *Puccinia graminis secalis* with *P. graminis tritici*.

Changes in environmental conditions affect perceptibly the degree of infection and virulence quotients of forms of rye stem rust but not their physiologic specialization. Pathogenically these forms appear to remain genetically constant under varying conditions of temperature and luminescence. L. W. Melander [5] observed yellow pustules in a brown culture of rye stem rust, supplied to him by the writers and grown by him under constant light (162 foot-candles) at 20° C. The yellow pustules also were present at the same time in the original culture grown by the writers under ordinary greenhouse conditions. This would tend to show the presence of color mutation in *Puccinia graminis secalis*, similar to that described by Newton and Johnson (7) for *P. graminis tritici*. Extensive tests proved the two varicolored cultures to be pathogenically identical, namely, *P. graminis secalis* form 7.

Many varieties of barley and all the differential varieties of wheat, as well as the oat differentials, were subjected to a thorough test with the *secalis* forms. The oat varieties were extremely resistant throughout. All the differential varieties of wheat reacted in like manner, except Little Club. This variety reacted heterogeneously to a few collections of *Puccinia graminis secalis* when first inoculated. In all these tests the presence of forms of *P. graminis tritici* was detected. In one instance, however, the presence of the wheat stem rust was not discovered until after many tests had been made. Its occurrence may therefore have resulted from a subsequent accidental contamination, and the moderate development of rust on Little Club evidently was entirely due to the rye rust. Unfortunately, this rust strain was

[5] Associate Pathologist, Division of Barberry Eradication, Bureau of Plant Industry.

destroyed before monosporous cultures could be obtained for further intensive analysis. However, reasonably pure cultures of forms 7 and 11 of *P. graminis secalis* recently have been identified that infected Little Club wheat in an indeterminate (mesothetic) manner, although all other wheat differentials definitely proved to be extremely resistant to these forms.

Between 30 and 40 varieties of barley belonging to the species *Hordeum vulgare* L., *H. intermedium* Kcke., *H. distichon* L., and *H. deficiens* Steud., reacted variously to the different tested forms of *Puccinia graminis secalis*. A detailed study of the reaction of barley varieties to the different cereal rusts is at present being completed.

SUMMARY

During the period from 1921 to 1931, inclusive, close to 150 collections of *Puccinia graminis secalis* were studied to determine the physiologic specialization within this rust variety.

Fourteen physiologic forms have been distinguished, and the characteristic parasitic behavior of each has been ascertained on several differential varieties of *Secale cereale*.

Because rye is cross-pollinated, the identification technic is more involved than in the case of stem rust of either wheat or oats, but apparently is not less certain.

Some of the physiologic forms identified occurred frequently and were widely distributed, others occurred rarely and in restricted areas.

Form 11 was isolated from the largest number of localities and was an important factor in the rye stem-rust epidemics of 1926, 1927, 1929, and 1931, occurring with approximately even frequency in all these years except 1927.

Although form 7 was collected almost as often as form 11, it appeared to be scattered over a greater geographic area and was isolated at different times for six, instead of nine, years of the present investigation. It was especially common in 1927.

Forms 1, 6, 10, and 13 were collected only once each; the remaining eight forms were isolated from two to ten times each.

The frequency of the occurrence of the different physiologic forms was not always coextensive with their distribution, nor was the prevalence of a given physiologic form paralleled by its virulence on the differential varieties.

The pathogenicity of the physiologic forms of *Puccinia graminis secalis* was but slightly and only temporarily affected by external conditions, such as temperature and light.

There seems to be strong circumstantial evidence of the occurrence of color mutations in the rye stem rust, but none so far of mutations in parasitic behavior.

The possibility of the origin of rye stem-rust forms through hybridization in the aecial stage is supported by the evidence of the production of new forms of wheat stem rust by crossing *Puccinia graminis secalis* with *P. graminis tritici*.

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LEAD ARSENATE POISONING IN CHICKENS[1]

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INTRODUCTION

During the spring of 1929 the veterinarians of the Florida experiment station staff were called upon to investigate numerous complaints from poultry owners who lived in areas of Florida in which lead arsenate spray was being used in combating the Mediterranean fruit fly. Since it was impossible to obtain information on the toxicity of lead arsenate to chickens, and there were few indications of poisoning in the greater number of cases, it was necessary to conduct tests with lead arsenate and lead arsenate spray solution to determine their effect on chickens.

The spray formula used by the United States Department of Agriculture in cooperation with the Florida State plant board in the campaign against the Mediterranean fruit fly was as follows: Lead arsenate, 8 pounds; brown sugar, 50 pounds; syrup, 10 gallons; and water sufficient to make 200 gallons. In all tests with poultry the solution was prepared according to this formula, and made up in 1-gallon quantities.

EXPERIMENTAL DATA

The tests to determine the effect of powdered lead arsenate on chickens were conducted first. Each chicken was assigned a number, weighed, and fed a definite quantity of lead arsenate powder. Observations were made several times daily after the feeding. In all cases the lead arsenate was given in capsules except to the birds receiving 1-ounce and 2-ounce doses, where it was necessary to add the powder to water and administer as a solution. Table 1 shows the quantity given each bird and the results obtained. Birds used as checks in adjacent pens were all alive and healthy at the termination of these tests.

To determine the effects of allowing chickens to have access to lead arsenate spray solution continually both as drink and food, the following tests were conducted.

Six frying-size chickens of mixed breeding, two of which weighed 2 pounds each and four of which weighed 3 pounds each, were placed in pen No. 1, July 1, 1929. These birds were allowed nothing to drink but lead arsenate spray solution. They were fed mash and scratch grain as usual. A gallon of the solution was made up daily and placed before them in an inverted type of fountain. This type of drinking fountain assured the consumption of the stronger solution, since lead arsenate has a tendency to settle to the bottom on standing. The six birds consumed approximately 1 quart daily. The test lasted for 60 days, ending September 1, 1929. At the end of the test period all the birds showed a normal gain in weight and were apparently normal in every other respect.

[1] Received for publication Jan. 19, 1932; issued September, 1932.

TABLE 1.—*Results of feeding various quantities of powdered lead arsenate to chickens*

Bird No.	Weight	Date fed lead arsenate	Quantity of lead arsenate fed	Effects
	Ounces		*Grains*	
311	52	June 21	20	Died June 29.
377	20			Lived.
381	20			Do.
345	40			Do.
392	48			Do.
342	56			Do.
384	18		40	Lived.
343	24			Died June 27.
329	28			Died July 1.
364	44			Lived.
393	48			Do.
382	56			Do.
338	24		60	Died July 1.
386	20			Died July 4.
369	20			Died July 6.
383	16	June 21	80	Died June 25.
74	24			Died June 29.
380	28			Lived.
348	36			Do.
53	48			Do.
62	76			Died July 18.
375	52		160	Died July 1.
376	72			Died July 2.
38	100			Died June 29.
310	16		240	Died June 24.
361	20			Died June 25.
368	24			Died June 24.
302	32	June 22	*Ounces* 1	Died July 2.
344	40			Died June 27.
359	40			Lived.
136	112		2	Died June 27.

Six apparently healthy Buff Leghorn hens were placed in pen No. 2 on July 3, 1929. These birds were allowed nothing to eat except scratch grain that had been soaked in lead arsenate spray solution. They were given a quart of fresh soaked scratch feed daily, but the quantity consumed was not determined since a great deal was wasted by their scratching and pecking it out onto the ground. This test extended over a 60-day period and ended September 3. All six birds were apparently in good health at the end of the 60 days. Birds used as checks in adjacent pens were all alive and apparently in good health at the termination of the tests.

DISCUSSION

In pen No. 1 each bird consumed approximately 13 grains of lead arsenate daily, a total consumption of 780 grains over a 60-day period, and showed no symptoms of poisoning.

Birds in pen No. 2, although given only scratch feed, apparently suffered no ill effects from consuming a small amount of lead arsenate daily for 60 days.

Of 31 birds fed powdered lead arsenate, 18 died from lead arsenate poisoning; of 6 fed 20 grains, 1 died; of 6 fed 40 grains, 2 died; of 3 fed 60 grains, 3 died; of 6 fed 80 grains, 3 died; of 3 fed 160 grains, 3 died; of 3 fed 240 grains, 3 died; of 3 fed 1 ounce, 2 died; and the only bird fed 2 ounces died. (Table 1.) Death was produced in 2 to 27 days, the average being 9 days. The cause of death in each case was determined by post-mortem examination.

The symptoms of the birds poisoned by lead arsenate were drowsiness, thirst, loss of appetite, diarrhea, and, in the last stages, cyanotic combs and wattles. A few of the birds passed several ascarids during the first 48 hours after they were fed the lead arsenate. On autopsy no differences were noted in the severity of the poisoning in those birds that passed ascarids and those that did not.

On post-mortem examination, birds fed the larger amounts of lead arsenate showed some of the material still in the crop and gizzard,

and in such birds necrotic areas were found in the crop lining. Other post-mortem lesions were dark congested liver, congested and hemorrhagic intestinal mucosa with blood-stained contents and a large amount of mucus. The kidneys were pale in color.

All birds used in these lead arsenate tests that were apparently healthy at the end of the experiment were used in other kinds of tests, and post-mortem examinations made on them during the next year did not reveal any lesions that were associated with lead arsenate poisoning.

Since it would seem reasonable to expect decreased egg production and loss of weight in hens on restricted diets such as were fed to those in pens No. 1 and 2, it was not deemed necessary to consider these factors in these tests.

In the Mediterranean fruit-fly campaign approximately 7 gallons of lead arsenate spray solution was used on an acre of citrus. The results of the experiments here reported indicate that 168 hens could consume this amount continually for 60 days and suffer no ill effects. It would seem, therefore, that it is impossible for chickens to consume enough solution or sprayed foliage in a sprayed grove to suffer from the effects of lead arsenate poisoning.

CONCLUSIONS

Lead arsenate will produce death in chickens when fed in large quantities.

There appeared to be no definite correlation between the weights of the birds and a lethal dose of lead arsenate.

Lead arsenate spray solution of the strength used in these tests is apparently not harmful to chickens when consumed with feed and water continually for 60 days. A bird may consume as much as 13 grains per day for 60 days without suffering any ill effects.

U. S. GOVERNMENT PRINTING OFFICE: 1932